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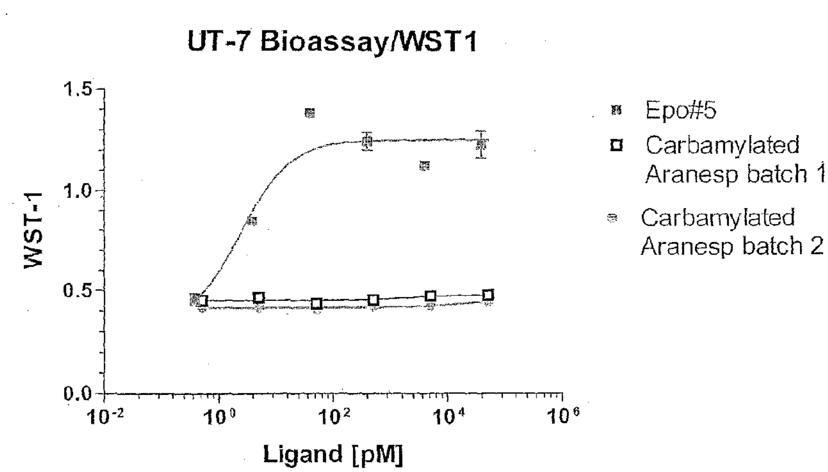
(54) Title: LONG ACTING TISSUE PROTECTIVE CYTOKINES FOR THE PROTECTION, RESTORATION, AND ENHANCEMENT OF RESPONSIVE CELLS, TISSUES AND ORGANS



(57) Abstract: Methods and compositions are provided for protecting or enhancing a responsive cell, tissue, organ or body part function or viability *in vivo, in situ* or *ex vivo* in mammals, including human beings, by systemic or local administration of a long acting tissue protective cytokine. In particular, the long acting tissue protective cytokines of the present invention relate to modified long acting erythropoietins exhibiting a tissue protective effect without an erythropoietic related activity.

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LONG ACTING TISSUE PROTECTIVE CYTOKINES FOR THE PROTECTION, RESTORATION, AND ENHANCEMENT OF RESPONSIVE CELLS, TISSUES AND ORGANS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority to US Provisional Application No. 60/549,675, entitled Tissue Protective Cytokines for the Protection, Restoration, and Enhancement of Responsive Cells, Tissues and Organs which is incorporated in its entirety by reference.

BACKGROUND OF THE INVENTION

For many years, the only clear physiological role of erythropoietin had been its control of the production of red blood cells. Recently, several lines of evidence suggest that erythropoietin, as a member of the cytokine superfamily, performs other important physiologic functions which are mediated through interaction with the erythropoietin receptor (erythropoietin-R). These actions include mitogenesis, modulation of calcium influx into smooth muscle cells and neural cells, production of erythrocytes, hyperactivation of platelets, production of thrombocytes, and effects on intermediary metabolism. It is believed that erythropoietin provides compensatory responses that serve to improve hypoxic cellular microenvironments as well as modulate programmed cell death caused by metabolic stress. Although studies have established that erythropoietin injected intracranially protects neurons against hypoxic neuronal injury, intracranial administration is an impractical and unacceptable route of administration for therapeutic use, particularly for normal individuals. Furthermore, previous studies of anemic patients given erythropoietin have concluded that peripherally-administered erythropoietin is not transported into the brain (Marti *et al.*, 1997, Kidney Int. 51:416-8; Juul et al., 1999, Pediatr. Res. 46:543-547; Buemi et al., 2000, Nephrol. Dial. Transplant. 15:422-433).

"Erythropoietin" is a glycoprotein hormone which in humans has a molecular weight of about 30-34 kDa. The mature protein has about 165 amino acids (SEQ. ID. NO. 5), and the oligosaccharide residues comprise about 40% of the weight of the molecule. Erythropoietin is more commonly associated with its effects on the bone marrow, *i.e.*, increased hematocrit (erythropoiesis), vasoconstriction (high blood pressure), hyperactivation of platelets, pro-coagulant activity, and increased production of thrombocytes. *See* U.S. Patent Nos. 4,703,008, 5,441,868, 5,547,933, 5,618,698, 5,621,080, 5,756,349, and 5,955,422. Erythropoietin can be obtained commercially, for example, under the trademarks of PROCRIT, available from Ortho Biotech Inc., Raritan, NJ, EPOGEN, available from Amgen, Inc., Thousand Oaks, CA, and NEORECORMON, erythropoietin beta, F. Hoffman-La Roche Ltd., Basel, Switzerland. Furthermore, a variety of host systems may be used for expression and production of recombinant erythropoietin, including, but not limited to, bacteria, yeast, insect, plant, and mammalian, including human, cell systems. For example, recombinant erythropoietin produced in bacteria, which do not glycosylate or sialylate the product, can be used to produce non-glycosylated forms of erythropoietin. Alternatively, recombinant erythropoietin can be produced in other systems that do glycosylate, *e.g.*, plants, and human cells.

Additionally, modified forms of erythropoietin have been previously disclosed as a means of enhancing the attributes of naturally occurring or recombinant human erythropoietin. Modified erythropoietins encompass chemical modifications and/or expression-system-mediated glycosylation modifications of naturally occurring, synthetic and recombinant forms of human and other mammalian erythropoietins. These modified erythropoietins include, but are not limited to, enhanced forms of erythropoietin such as those with altered amino acids at the carboxy terminus described in U.S. Patent 5,457,089 and in U.S. Patent No. 4,835,260; erythropoietin isoforms with various numbers of sialic acid residues per molecule, such as described in U.S. Patent 5,856,298; polypeptides described in U.S. Patent 4,703,008; agonists described in U.S. Patent 5,767,078; peptides which bind to the erythropoietin receptor as described in U.S. Patents 5,773,569 and 5,830,851; and small-molecule mimetics as described in U.S. Patent 5,835,382.

The present invention relates to tissue protective cytokines generated by the chemical modification of erythropoietins, and in particular long acting erythropoietins, and

their uses for protecting, maintaining, enhancing, or restoring erythropoietin-responsive cells and associated cells, tissues and organs *in situ* as well as *ex vivo*, and to delivery of a tissue protective cytokine across an endothelial cell barrier for the purpose of protecting and enhancing erythropoietin-responsive cells and associated cells, tissues and organs distal to the vasculature, or to carry associated molecules across an endothelial cell barrier.

BRIEF SUMMARY OF THE INVENTION

In one embodiment of the present invention a long acting erythropoietin or a long acting tissue protective cytokine is used for the preparation of a pharmaceutical composition for protection against an injury or restoration of function following the injury to responsive mammalian cells, tissue or organ. In a further embodiment, the responsive mammalian cells comprise neuronal, brain, spinal cord, retinal, muscle, heart, lung, liver, kidney, small intestine, adrenal cortex, adrenal medulla, capillary, endothelial, testes, ovary, endometrial, or stem cells. The cells may further comprise photoreceptor, ganglion, bipolar, horizontal, amacrine, Müller, myocardium, pace maker, sinoatrial node, sinus node, atrioventricular node, bundle of His, hepatocyte, stellate, Kupffer, mesangial, goblet, intestinal gland, enteral, endocrine, glomerulosa, fasciculate, reticularis, chromaffin, pericyte, Leydig, Sertoli, sperm, Graafian follicles, primordial follicles, endometrial stroma, and endometrial cells.

In another embodiment the long acting erythropoietin or long acting tissue protective cytokine is used for the preparation of a pharmaceutical composition for protection against an injury such as a seizure disorder, multiple sclerosis, stroke, hypotension, cardiac arrest, ischemia, myocardial infarction, inflammation, age-related loss of cognitive function, radiation damage, cerebral palsy, a neurodegenerative disease, Alzheimer's disease, Parkinson's disease, Leigh disease, AIDS dementia, memory loss, amyotrophic lateral sclerosis, alcoholism, mood disorder, anxiety disorder, attention deficit disorder, schizophrenia, autism, Creutzfeld-Jakob disease, brain or spinal cord trauma or ischemia, heart-lung bypass, chronic heart failure, macular degeneration, toxin induced neuropathy, diabetic neuropathy, diabetic retinopathy, glaucoma, retinal ischemia, or retinal trauma.

In yet another embodiment, the long acting erythropoietin used for the preparation of a pharmaceutical composition is selected from the group consisting of a chemically modified long acting erythropoietin and a recombinant long acting erythropoietin. Also, in an embodiment of the invention, the long acting tissue protective cytokine used for the preparation of the pharmaceutical composition lacks at least one activity selected from the group consisting of increasing hematocrit, vasoconstriction, hyperactivating platelets, pro-coagulant activity and increasing production of thrombocytes. Further, the long acting tissue protective cytokine is selected from the group consisting of a chemically modified long acting erythropoietin and a recombinant long acting erythropoietin. In a preferred embodiment the chemically modified long acting erythropoietin is selected from the group consisting of

i. A long acting erythropoietin having at least one or more oxidized carbohydrates;
ii. A chemically reduced long acting erythropoietin having at least one or more oxidized carbohydrates;
iii. A long acting erythropoietin having at least one or more modified arginine residues;
iv. A long acting erythropoietin having at least one or more modified lysine residues
v. A long acting erythropoietin having at least one modification of the N-terminal amino group of the erythropoietin molecule;
vi. A long acting erythropoietin having at least a modified tyrosine residue;
vii. A long acting erythropoietin having at least a modified aspartic acid or glutamic acid residue;
viii. A long acting erythropoietin having at least a modified tryptophan residue;
ix. A long acting erythropoietin having at least one amino acid removed;
x. A long acting erythropoietin having at least one opening of at least one of the cystine linkages in the long acting erythropoietin molecule; and
xi. A truncated long acting erythropoietin.

Additionally, the long acting recombinant erythropoietin is a long acting erythropoietin mutein of one or more altered amino acid residue between position 11 to 15 of SEQ ID NO:5 [SEQ ID NO:1], position 44 to 51 of SEQ ID NO 5 [SEQ ID NO:2], position 100-

108 of SEQ ID NO 5 [SEQ ID NO:3], or position 146-151 of SEQ ID NO 5 [SEQ ID NO:4]. In a preferred embodiment the long acting erythropoietin is a novel erythropoiesis stimulating protein, more preferably it is a novel erythropoiesis protein that has additional n-linked carbohydrate chains at erythropoietin amino acid residues 30 and 38. The novel erythropoiesis stimulating protein may be chemically modified or mutated, and preferably, the novel erythropoiesis stimulating protein has at least one carbamylated lysine residue. Furthermore, the long acting erythropoietin can be a diglycosylated and pegylated erythropoietin and this diglycosylated and pegylated erythropoietin may be chemically modified or mutated.

The present invention is further directed to a method for protecting or maintaining the viability of a responsive mammalian cell, tissue or organ by administering to a mammal a pharmaceutical composition including a long acting erythropoietin or a long acting tissue protective cytokine. Additionally, in another embodiment, the invention is directed to a method of protecting or maintaining the viability of *ex vivo* responsive mammalian cell, tissue or organ by contacting the cell, tissue, or organ with a pharmaceutical composition including a long acting erythropoietin or a long acting tissue protective cytokine. In a further embodiment, the responsive mammalian cells of the above methods include neuronal, brain, spinal cord, retinal, muscle, heart, lung, liver, kidney, small intestine, adrenal cortex, adrenal medulla, capillary, endothelial, testes, ovary, endometrial, or stem cells. The cells may further include photoreceptor, ganglion, bipolar, horizontal, amacrine, Müller, myocardium, pace maker, sinoatrial node, sinus node, atrioventricular node, bundle of His, hepatocyte, stellate, Kupffer, mesangial, goblet, intestinal gland, enteral, endocrine, glomerulosa, fasciculate, reticularis, chromaffin, pericyte, Leydig, Sertoli, sperm, Graafian follicles, primordial follicles, endometrial stroma, and endometrial cells.

In another embodiment the method of treatment using long acting erythropoietin or long acting tissue protective cytokine protects against or maintains the viability of cells after an injury such as a seizure disorder, multiple sclerosis, stroke, hypotension, cardiac arrest, ischemia, myocardial infarction, inflammation, age-related loss of cognitive function, radiation damage, cerebral palsy, a neurodegenerative disease, Alzheimer's disease, Parkinson's disease, Leigh disease, AIDS dementia, memory loss, amyotrophic

lateral sclerosis, alcoholism, mood disorder, anxiety disorder, attention deficit disorder, schizophrenia, autism, Creutzfeld-Jakob disease, brain or spinal cord trauma or ischemia, heart-lung bypass, chronic heart failure, macular degeneration, toxin induced neuropathy, diabetic neuropathy, diabetic retinopathy, glaucoma, retinal ischemia, or retinal trauma.

In yet another embodiment, the long acting erythropoietin used in the method of treatment is selected from the group consisting of a chemically modified long acting erythropoietin and a recombinant long acting erythropoietin. Also, in an embodiment of the invention, the long acting tissue protective cytokine used for the method of treatment lacks at least one activity selected from the group consisting of increasing hematocrit, vasoconstriction, hyperactivating platelets, pro-coagulant activity and increasing production of thrombocytes. Further, the long acting tissue protective cytokine is selected from the group consisting of a chemically modified long acting erythropoietin and a recombinant long acting erythropoietin. In a preferred embodiment the chemically modified long acting erythropoietin is selected from the group consisting of

i. A long acting erythropoietin having at least one or more oxidized carbohydrates;

ii. A chemically reduced long acting erythropoietin having at least one or more oxidized carbohydrates;

iii. A long acting erythropoietin having at least one or more modified arginine residues;

iv. A long acting erythropoietin having at least one or more modified lysine residues

v. A long acting erythropoietin having at least one modification of the N-terminal amino group of the erythropoietin molecule;

vi. A long acting erythropoietin having at least a modified tyrosine residue;

vii. A long acting erythropoietin having at least a modified aspartic acid or glutamic acid residue;

viii. A long acting erythropoietin having at least a modified tryptophan residue;

ix. A long acting erythropoietin having at least one amino acid removed;

x. A long acting erythropoietin having at least one opening of at least one of the cystine linkages in the long acting erythropoietin molecule; and

xi. A truncated long acting erythropoietin.

Additionally, the long acting recombinant erythropoietin is a long acting erythropoietin mutein of one or more altered amino acid residue between position 11 to 15 of SEQ ID NO:5 [SEQ ID NO:1], position 44 to 51 of SEQ ID NO 5 [SEQ ID NO:2], position 100-108 of SEQ ID NO 5 [SEQ ID NO:3], or position 146-151 of SEQ ID NO 5 [SEQ ID NO:4]. In a preferred embodiment the long acting erythropoietin used in the method of treatment is a novel erythropoiesis stimulating protein, more preferably it is a novel erythropoiesis protein that has additional n-linked carbohydrate chains at erythropoietin amino acid residues 30 and 38. The novel erythropoiesis stimulating protein may be chemically modified or mutated, and preferably, the novel erythropoiesis stimulating protein has at least one carbamylated lysine residue. Furthermore, the long acting erythropoietin can be a diglycosylated and pegylated erythropoietin and this diglycosylated and pegylated erythropoietin may be chemically modified or mutated.

In another embodiment of the current invention, a composition for transporting a molecule via transcytosis across an endothelial cell barrier is provided for. The composition includes the molecule in association with a long acting erythropoietin or long acting tissue protective cytokine. In another embodiment, the molecule in association with the long acting erythropoietin or long acting tissue protective cytokine may be a hormone, neurotrophic factor, antibiotic, antiviral, antifungal, peptide radiopharmaceutical, antisense drug, antibody, antiviral, pharmaceutical, or anti-cancer agent.

In yet another embodiment, the long acting erythropoietin used in the composition is selected from the group consisting of a chemically modified long acting erythropoietin and a recombinant long acting erythropoietin. Also, in an embodiment of the invention, the long acting tissue protective cytokine used for the composition lacks at least one activity selected from the group consisting of increasing hematocrit, vasoconstriction, hyperactivating platelets, pro-coagulant activity and increasing production of thrombocytes. Further, the long acting tissue protective cytokine is selected from the group consisting of a chemically modified long acting erythropoietin and a recombinant long acting erythropoietin. In a preferred embodiment the chemically modified long acting erythropoietin is selected from the group consisting of

i. A long acting erythropoietin having at least one or more oxidized carbohydrates;

ii. A chemically reduced long acting erythropoietin having at least one or more oxidized carbohydrates;

iii. A long acting erythropoietin having at least one or more modified arginine residues;

iv. A long acting erythropoietin having at least one or more modified lysine residues

v. A long acting erythropoietin having at least one modification of the N-terminal amino group of the erythropoietin molecule;

vi. A long acting erythropoietin having at least a modified tyrosine residue;

vii. A long acting erythropoietin having at least a modified aspartic acid or glutamic acid residue;

viii. A long acting erythropoietin having at least a modified tryptophan residue;

ix. A long acting erythropoietin having at least one amino acid removed;

x. A long acting erythropoietin having at least one opening of at least one of the cystine linkages in the long acting erythropoietin molecule; and

xi. A truncated long acting erythropoietin.

Additionally, the long acting recombinant erythropoietin is a long acting erythropoietin mutein of one or more altered amino acid residue between position 11 to 15 of SEQ ID NO:5 [SEQ ID NO:1], position 44 to 51 of SEQ ID NO 5 [SEQ ID NO:2], position 100-108 of SEQ ID NO 5 [SEQ ID NO:3], or position 146-151 of SEQ ID NO 5 [SEQ ID NO:4]. In a preferred embodiment the long acting erythropoietin used in the composition is a novel erythropoiesis stimulating protein, more preferably it is a novel erythropoiesis protein that has additional n-linked carbohydrate chains at erythropoietin amino acid residues 30 and 38. The novel erythropoiesis stimulating protein may be chemically modified or mutated, and preferably, the novel erythropoiesis stimulating protein has at least one carbamylated lysine residue. Furthermore, the long acting erythropoietin can be a diglycosylated and pegylated erythropoietin and this diglycosylated and pegylated erythropoietin may be chemically modified or mutated.

Another embodiment of the current invention contemplated is a pharmaceutical composition made of a chemically modified long acting erythropoietin having at least one responsive cellular protective activity such as protecting, maintaining, enhancing or restoring the function or viability of a responsive mammalian cell, tissue or organ. The responsive mammalian cells may include neuronal, brain, spinal cord, retinal, muscle, heart, lung, liver, kidney, small intestine, adrenal cortex, adrenal medulla, capillary, endothelial, testes, ovary, endometrial, or stem cells. In another embodiment, the cells further include photoreceptor, ganglion, bipolar, horizontal, amacrine, Müller, myocardium, pace maker, sinoatrial node, sinus node, atrioventricular node, bundle of His, hepatocyte, stellate, Kupffer, mesangial, goblet, intestinal gland, enteral endocrine, glomerulosa, fasciculate, reticularis, chromaffin, pericyte, Leydig, Sertoli, sperm, Graafian follicles, primordial follicles, endometrial stroma, and endometrial cells.

In another embodiment of the the pharmaceutical composition, the chemically modified long acting erythropoietin lacks at least one activity such as increasing hematocrit, vasoconstriction, hyperactivating platelets, pro-coagulant activity and increasing production of thrombocytes. The chemically modified long acting erythropoietin may further be selected from the group consisting of

i. A long acting erythropoietin having at least one or more oxidized carbohydrates;

ii. A chemically reduced long acting erythropoietin having at least one or more oxidized carbohydrates;

iii. A long acting erythropoietin having at least one or more modified arginine residues;

iv. A long acting erythropoietin having at least one or more modified lysine residues

v. A long acting erythropoietin having at least one modification of the N-terminal amino group of the erythropoietin molecule;

vi. A long acting erythropoietin having at least a modified tyrosine residue;

vii. A long acting erythropoietin having at least a modified aspartic acid or glutamic acid residue;

viii. A long acting erythropoietin having at least a modified tryptophan residue;

ix. A long acting erythropoietin having at least one amino acid removed;

x. A long acting erythropoietin having at least one opening of at least one of the cystine linkages in the long acting erythropoietin molecule; and

xi. A truncated long acting erythropoietin.

In another embodiment the chemically modified erythropoietin of the pharmaceutical composition may further be modified by altering one or more amino acid residues between position 11 to 15 of SEQ ID NO:5 [SEQ ID NO:1], position 44 to 51 of SEQ ID NO 5 [SEQ ID NO:2], position 100-108 of SEQ ID NO 5 [SEQ ID NO:3], or position 146-151 of SEQ ID NO 5 [SEQ ID NO:4]. Preferably the chemically modified long acting erythropoietin of the pharmaceutical composition is a novel erythropoiesis stimulating protein, more preferably, the novel erythropoiesis stimulating protein has additional n-linked carbohydrate chains at erythropoietin amino acid residues 30 and 38. The novel erythropoiesis stimulating protein may have at least one carbamylated lysine residue. Further, the chemically modified long acting erythropoietin of the pharmaceutical compositions may be a diglycosylated and pegylated erythropoietin.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows that carbamylated NESP is non erythropoietic (*i.e.*, does not support proliferation) within an erythroleukemia UT-7 bioassay.

Figure 2 shows that carbamylated NESP protects primary hippocampal neuronal cultures against NMDA toxicity in an *in vitro* model of neuroprotection.

Figure 3 shows that treatment with carbamylated NESP reduces the infarct size resulting from a Middle Cerebral Artery Occlusion (MCAO) injury model in rats.

Figure 4 shows that rats treated with carbamylated NESP in the MCAO injury model score higher on the DeRyck sensor-motor behavioral test than placebo-treated MCAO injured rats.

DETAILED DESCRIPTION OF THE INVENTION

The various methods of the invention utilize a pharmaceutical composition that at least includes a long acting erythropoietin or a long acting tissue protective cytokine at an effective amount for the particular route of administration, time of administration, and

duration of exposure to exert positive effects or benefits on responsive cells within a mammalian body. Where the target cell, tissues, or organs of the intended therapy require an long acting erythropoietin or a long acting tissue protective cytokine to cross an endothelial cell barrier, the pharmaceutical composition includes a long acting erythropoietin or a long acting tissue protective cytokine at a concentration which is capable, after crossing the endothelial cell barrier, of exerting its desirable effects upon the responsive cells. Molecules capable of exerting an effect interacting with the erythropoietin receptor and modulating the activity of the receptor, herein referred to generally as erythropoietin or erythropoietin receptor activity modulators, are useful in the context of the present invention. These molecules may be, for example, naturally-occurring, synthetic, or recombinant forms of erythropoietin molecules, as described below, or other molecules which may not necessarily resemble erythropoietin in any manner, except to modulate erythropoietin responsive cell activity, as described herein.

II. Long Acting Erythropoietin

Various modified forms of erythropoietin having an extended half-life – longer than the half-life of naturally occurring or recombinant human erythropoietin – have been described previously in an effort to provide a preferred dosing schedule for patients – fewer visits to the physician for intravenous or subcutaneous injections. These "Long Acting Erythropoietins" in general are modified forms of erythropoietin having an extended half-life due to increased sialic acid residues as taught in U.S. Patent 5,856,298, the addition of sugars as taught in EP0640619, the addition of polyethylene glycol (PEG) residues as taught in WO0102017, WO0032772, and U.S. Patent Application Serial No. 200301666566, the additions of proteins through fusion with erythropoietin as taught in U.S. Patent Application Serial Nos. 20040009902, 20030124115, and 20030113871 as well as U.S. Patent No. 6,242,570, the modification of the naturally occurring glycosylation pattern of either recombinant or naturally occurring human erythropoietin as taught in EP0640619, PCT application number US/94/02957 and U.S. Patent Application Serial No. 20030077753, and/or mutations as taught in U.S. Patent Application Serial No. 20020081734. Further, they may include modified erythropoietins include diglycosylated and pegylated erythropoietin conjugates taught in the following patent applications WO0102017, EP1064951, EP1345628, WO03029291, US2003077753, US20030120045

and U.S. Patent Nos. 6,583,272 and 6,340,742. Commercial examples of such long acting erythropoietins are darbepoietin alpha, ARANESP, available from Amgen Inc., Thousand Oaks, California, USA, CERA (Continuous Erythropoiesis Receptor Activator) currently in clinical trials conducted by F. Hoffmann-La Roche, Basel, Switzerland, and the diglycosylated and pegylated erythropoietins taught in WO03029291. Each of these long acting erythropoietins are incorporated within the current invention.

Specifically, ARANESP is a long acting erythropoietin referred to as a novel erythropoietin stimulating protein (NESP). As is further detailed in WO94/02957 and EP 0640619, NESPs are hyperglycosylated erythropoietin analogs wherein the amino acid sequence of rHuEPO is modified to provide for additional N-linked or O-Linked carbohydrate chains. This is accomplished by mutating the amino acid sequence of erythropoietin by substituting an asparagine, serine or threonine to generate additional glycosylation sites (the amino acid sequence asparagine (ASN)-X-serine(SER)/threonine(THR) (where X is any amino acid except proline) signifies a glycosylation site within erythropoietin). Specifically, ARANESP is an erythropoietin that has five modifications (Ala30Asn, His32Thr, Pro87Val, Trp88Asn, and Pro90Thr) to generate two additional N-linked carbohydrate chains at amino acid residues 30 and 88 within the amino acid sequence of human erythropoietin (see PCT Application No. US94/02957, herein incorporated by reference in its entirety). These modifications result in an erythropoietin that has a serum half-life three times longer than that of erythropoietin alpha, thereby allowing for less frequent dosing. Aditionally, ARANESP differs from epoetin alfa by having a lower isoelectric point, an increased carbohydrate content (52% vs. 40%), a higher molecular weight (37,100 vs. 30,400), and lower receptor-binding affinity.

Similarly, the diglycosylated and pegylated erythropoietin attempts to provide erythropoietin with enhanced characteristics, such as an extended half-life. As is taught in WO03/029291, incorporated herein by reference, the naturally occurring N-glycosylation sites at Asn 38 and Asn 83 of erythropoietin are maintained in these long acting erythropoietins. However, the N-glycosylation site at Asn 24 is eliminated without mutation and the diglycosylated and pegylated erythropoietin compound is not glycosylated prior to Cys29. This is accomplished by permitting the fragment of EPO

from amino acid 29-165/166 to be produced recombinantly in eukaryotic cells (C-terminal fragment) and generating in vitro a chemically synthesized fragment of EPO from amino acid 1-28 (N-terminal fragment). Poly(ethylene glycol) groups, including but not limited to a 30 kDa methoxy-polyethylene glycol polymer, may be attached at the N-terminal amino group and/or the ε-amino group of Lys20 of the chemically synthesized fragment prior to ligating the C-terminal fragment with the N-terminal fragment.

II. Long Acting Tissue Protective Cytokines

Erythropoietin has been attributed with tissue protective effects as previously disclosed in PCT Patent Application PCT/US00/10019, entitled Modulation of Excitable Tissue Function by Peripherally Administered Erythropoietin, which is incorporated herein by reference in its entirety, and the long acting erythropoietins exhibit these tissue protective activities as well. However, given the longer circulating half-lives of long acting erythropoietins, their use as tissue protectants is complicated by possible erythropoietic side effects such as polycythemia, hypertension, and thrombotic events, all of which are currently noted on the warning label for ARANESP. Therefore, a long acting erythropoietin that possesses primarily or solely tissue protective activity is the object of the present invention. "Long Acting Tissue Protective Cytokine" refers to cytokines that exhibit tissue protective effects and lack one or more of erythropoietin's effects on the bone marrow – increased hematocrit (erythropoiesis), hyperactivation of platelets, procoagulant activity, and increased production of thrombocytes – or on the vasculature – vasoconstriction (high blood pressure). The long acting tissue protective cytokines may be chemically modified or recombinant forms of long acting erythropoietin. With both the chemically modified and recombinant long acting erythropoietins, preferably the alterations occur within the erythropoietin amino acid sequence (SEQ ID NO:5) within four functional domains which affect receptor binding: VLQRY (SEQ ID NO:1) and/or TKVNFYAW (SEQ ID NO:2) and/or SGLRSLTTL (SEQ ID NO:3) and/or SNFLRG (SEQ ID NO:4).

A. Chemical Modifications.

The Long Acting Tissue Protective Cytokines may consist of chemically modified long acting erythropoietins. The Long Acting Erythropoietin may be modified by the guanidation, amidination, carbamylation (carbamoylation), triphenylation, acetylation, succinylation, nitration, or modification of the N-terminal amino group, arginine, lysine, tyrosine, tryptophan, or cysteine residues or aspartic, glutamic, or C-terminal carboxyl groups of the long acting erythropoietin; additionally, procedures such as limited proteolysis may be employed to further modify the long acting erythropoietin, as disclosed in U.S. Patent Application No. 10/188,905, entitled Tissue Protective Cytokines for the Protection, Restoration, and Enhancement of Erythropoietin Responsive Cells, Tissues and Organs; and PCT Patent Application No. PCT/US01/49479 entitled Protection, Restoration, and Enhancement of Erythropoietin Cells, Tissues and Organs incorporated herein by reference. At least one to all of a particular amino acid residue in a long acting erythropoietin may be modified using the above processes. For example, anywhere from one to all eight of the lysine amino acid residues within the long acting erythropoien may be modified through carbamylation in order to achieve a long acting tissue protective cytokine, *i.e.*, a long acting tissue protective cytokine that lacks one or more of erythropoeitin's effects on the bone marrow or vaculature. Preferably, these chemical modifications affect the basic amino acids, arginine and lysine, within the recognized receptor regions. Additionally, the areas of the molecule surrounding these receptor regions may be chemically modified as well to affect the kinetics or receptor binding properties of the molecule. This produces long acting tissue protective cytokines which maintain an adequate level of activities for specific organs and tissues but not for others, such as erythrocytes (e.g., Satake et al; 1990, *Biochim. Biophys. Acta* 1038:125-9; incorporated herein by reference in its entirety). One non-limiting example as described herein below is the modification of long acting erythropoietin arginine residues by reaction with a glyoxal such as phenylglyoxal (according to the protocol of Takahashi, 1977, *J. Biochem.* 81:395-402). Such long acting tissue protective cytokine molecules are further disclosed below.

A long acting erythropoietin for the aforementioned uses may have at least one or more modified lysine or arginine residues. For example, the modified long acting erythropoietin may comprise an R-glyoxal moiety on the one or more arginine residues, where R may be an aryl, heteroaryl, lower alkyl, lower alkoxy, or cycloalkyl group, or an

alpha-deoxyglycitolyl group. As used herein, the term lower "alkyl" means a straight- or branched-chain saturated or unsaturated aliphatic hydrocarbon group preferably containing 1-6 carbon atoms. Representative of such groups are methyl, ethyl, isopropyl, isobutyl, butyl, pentyl, hexyl and the like. The term "alkoxy" means a lower alkyl group as defined above attached to the remainder of the molecule by oxygen. Examples of alkoxy include methoxy, ethoxy, propoxy, isopropoxy and the like. The term "cycloalkyl" refers to cyclic alkyl groups with from three to up to about 8 carbons, including for example cyclopropyl, cyclobutyl, cyclohexyl and the like. The term aryl refers to phenyl and naphthyl groups. The term heteroaryl refers to heterocyclic groups containing 4-10 ring members and 1-3 heteroatoms selected from the group consisting of oxygen, nitrogen and sulfur. Examples include but are not limited to isoxazolyl, phenylisoxazolyl, furyl, pyrimidinyl, quinolyl, tetrahydroquinolyl, pyridyl, imidazolyl, pyrrolidinyl, 1,2,4-triazoylyl, thiazolyl, thienyl, and the like. The R group may be substituted, as for example the 2,3,4-trihydroxybutyl group of 3-deoxyglucosone. Typical examples of R-glyoxal compounds are glyoxal, methylglyoxal, 3-deoxyglucosone, hydroxyl phenylglyoxal, and phenylglyoxal. Preferred R-glyoxal compounds are methylglyoxal or phenylglyoxal. An exemplary method for such modification may be found in Werber et al., 1975, Isr. J. Med. Sci. 11(11): 1169-70, using phenylglyoxal.

In a further example, at least one arginine residue of a long acting erythropoietin may be modified by reaction with a vicinal diketone such as 2,3-butanedione, cyclohexanedione, or 1,2-cyclohexanedione, preferably in ca. 50 millimolar borate buffer at pH 8-9. A procedure for the latter modification with 2,3-butanedione may be carried out in accordance with Riordan, 1973, Biochemistry 12(20): 3915-3923; and that with cyclohexanone according to Patthy et al., 1975, J. Biol. Chem 250(2): 565-9.

A long acting tissue protective cytokine of the invention may comprise at least one or more modified lysine residues or a modification of the N-terminal amino group of the long acting erythropoietin molecule, such modifications as those resulting from reaction of the lysine residue with an amino-group-modifying agent. For example, long acting erythropoietin, may be modified by acetylation, carbamylation, succinylation, oxidation and subsequent carboxymethyllysination, among other methods, to modify amino groups.

In a non-limiting example, a long acting tissue protective cytokine may be generated by carbamylating a long acting erythropoietin with recrystallized potassium cyanate in borate buffer, after which thorough dialysis is performed.

Likewise, an aforementioned long acting erythropoietin may be succinylated by reaction with succinic anhydride, followed by dialysis to form a tissue protective cytokine of the present invention.

In yet another embodiment, a long acting tissue protective cytokine may be generated by reacting a long acting erythropoietin with acetic anhydride in phosphate buffer to acetylate the erythropoietin. This reaction may be stopped by dialysis against water. The method is described in Satake et al, (1990). Chemical modification of erythropoietin: an increase in in-vitro activity by guanidination. Biochimica et Biophysica Acta. 1038: 125-129.

In another embodiment, the long acting tissue protective cytokines are N^{ε}-(carboxymethyl)lysine (CML) adducts from long acting erythropoietin prepared by reaction with glyoxylic acid and $NaBH_3CN$ in sodium phosphate buffer, followed by dialysis. Akhtar et al., (1999) Conformational study of N^{ε}-(carboxymethyl)lysine adducts of recombinant a-crystallins. Current Eye Research, 18: 270-276.

In another embodiment, a long acting tissue protective cytokine is generated by modifying the lysine residues of long acting erythropoietin by reaction with glyoxal derivatives, such as reaction with glyoxal, methylglyoxal and 3-deoxyglucosone to form alpha-carboxyalkyl derivatives. Examples include reaction with glyoxal to form a carboxymethyllysine residue as in Glomb and Monnier, 1995, J. Biol. Chem. 270(17):10017-26, or with methylglyoxal to form a (1-carboxyethyl)lysine residue as in Degenhardt et al., 1998, Cell. Mol. Biol. (Noisy-le-grand) 44(7):1139-45. The modified lysine residue further may be chemically reduced. For example, the long acting erythropoietin may be biotinylated via lysine groups by reacting the long acting erythropoietin with D-biotinoyl-ε-aminocaproic acid-N-hydroxysuccinimide ester and removing unreacted biotin by gel filtration on a Centricon 10 column, as described by Wojchowski and Caslake, 1989, Blood 74(3):952-8. In this paper, the authors use three

different methods of biotinylating erythropoietin, any of which may be used for the preparation of the long acting tissue protective cytokines for the uses herein. Biotin may be added to (1) the sialic acid moieties (2) carboxylate groups or (3) amino groups.

In another preferred embodiment, the lysine may be reacted with an aldehyde or reducing sugar to form an imine, which may be stabilized by reduction as with sodium cyanoborohydride to form an N-alkylated lysine residue such as glucitolyl lysine, or which in the case of reducing sugars may be stabilized by Amadori or Heyns rearrangement to form an alpha-deoxy alpha-amino sugar such as alpha-deoxy-alpha-fructosyllysine residue in the long acting erythropoietin molecule. As an example, preparation of a fructosyllysine-modified protein by incubation with 0.5 M glucose in sodium phosphate buffer at pH 7.4 for 60 days is described by Makita et al., 1992, J. Biol. Chem. 267:5133-5138. In another example, the lysine group may be carbamylated, such as by virtue of reaction with cyanate ion, or alkyl- or aryl-carbamylated or -thiocarbamylated with an alkyl- or aryl-isocyanate or -isothiocyanate, or it may be acylated by a reactive alkyl- or arylcarboxylic acid derivative, such as by reaction with acetic anhydride or succinic anhydride or phthalic anhydride. Exemplary are the modification of lysine groups with 4-sulfophenylisothiocyanate or with acetic anhydride, both as described in Gao et al., 1994, Proc Natl Acad Sci USA 91(25):12027-30. Lysine groups may also be trinitrophenyl modified by reaction with trinitrobenzenesulfonic acid or preferably its salts.

At least one tyrosine residue of a long acting erythropoietin may be modified in an aromatic ring position by an electrophilic reagent, such as by nitration or iodination to generate a long acting tissue protective cytokine. By way of non-limiting example, long acting erythropoietin may be reacted with tetranitromethane (Nestler et al., 1985, J. Biol. Chem. 260(12):7316-21; or iodinated. For example, iodination with NaI and IODO-GEN Pre-Coated Iodination Tube (Pierce, 28601), may be carried out using a long acting erythropoietin in sodium phosphate buffer.

At least an aspartic acid or a glutamic acid residue of a long acting erythropoietin may be modified, such as by reaction with a carbodiimide followed by reaction with an amine such as but not limited to glycinamide. The same reaction may be carried out with the C-terminal carboxylic acid moiety.

In another example, a tryptophan residue of a long acting erythropoietin may be modified, such as by reaction with n-bromosuccinimide or n-chlorosuccinimide, following methods such as described in Josse et al., Chem Biol Interact 1999 May 14;119-120.

In yet another example, a long acting tissue protective cytokine may be prepared by removing at least one amino group of a long acting erythropoietin, such may be achieved by reaction with ninhydrin followed by reduction of the subsequent carbonyl group by reaction with borohydride.

In still a further example, a long acting tissue protective cytokine is provided that has at least an opening of at least one of the cystine linkages in the long acting erythropoietin molecule by reaction with a reducing agent such as dithiothreitol, followed by reaction of the subsequent sulfhydryls with iodoacetamide, iodoacetic acid or another electrophile to prevent reformation of the disulfide linkages. As noted above, alternatively or in combination, disulfide linkages may be abolished by altering a cysteine molecule that participates in the actual cross-link or at least one other amino acid residue that results in the inability of the long acting erythropoietin to form at least one of the disulfide linkages present in the native molecule.

A long acting tissue protective cytokine may be prepared by subjecting a long acting erythropoietin to a limited chemical proteolysis that targets specific residues, for example, to cleave after tryptophan residues. Such resulting erythropoietin fragments are embraced herein. Any of the foregoing modifications may be performed alone or in combination with one or more of the other modifications disclosed herein.

B. Recombinant Long Acting Erythropoietins.

Additionally, the Long Acting Tissue Protective Cytokines may consist of recombinant long acting erythropoietin. The recombinant long acting erythropoietin may consist of erythropoietin muteins. The disclosed mutations to long acting erythropoietin may include substitutions, deletions, including internal deletions, additions, including additions yielding fusion proteins, or conservative substitutions of amino acid residues

within and/or adjacent to the amino acid sequence, but that result in a "silent" change, and non-conservative amino acid changes and larger insertions and deletions, as previously disclosed in PCT/US03/20964 entitled Recombinant Tissue Protective Cytokines and Encoding Nucleic Acids Thereof for Protection, Restoration, and Enhancement of Responsive Cells, Tissues, and Organs.

Either conservative or non-conservative amino acid substitutions can be made at one or more amino acid residues. Both conservative and non-conservative substitutions can be made. Conservative replacements are those that take place within a family of amino acids that are related in their side chains. Genetically encoded amino acids can be divided into four families: (1) acidic = aspartate, glutamate; (2) basic = lysine, arginine, histidine; (3) nonpolar = alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan; and (4) uncharged polar = glycine, asparagine, glutamine, cysteine, serine, threonine, tyrosine. In similar fashion, the amino acid repertoire can be grouped as (1) acidic = aspartate, glutamate; (2) basic = lysine, arginine, histidine, (3) aliphatic = glycine, alanine, valine, leucine, isoleucine, serine, threonine, with serine and threonine optionally be grouped separately as aliphatic-hydroxyl; (4) aromatic = phenylalanine, tyrosine, tryptophan; (5) amide = asparagine, glutamine; and (6) sulfur - containing = cysteine and methionine. (See, for example, Biochemistry, 4th ed., Ed. by L. Stryer, WH Freeman and Co.: 1995).

Alternatively, mutations can be introduced randomly along all or part of the coding sequence of a recombinant long acting tissue protective cytokine, such as by saturation mutagenesis, and the resultant mutants can be screened for biological activity to identify mutants that retain activity. Following mutagenesis, the encoded protein can be expressed recombinantly and the activity of the recombinant long acting tissue protective cytokine can be determined.

Further to the above-mentioned long acting erythropoietin modifications useful herein, the following discussion expands on the various recombinant long acting tissue protective cytokines of the invention. The following long acting erythropoietin muteins are useful for the purposes described herein, and may be provided in a pharmaceutical composition for the methods herein. Notably, the long acting erythropoietins possess the same amino acid sequence of recombinant erythropoietin (SEQ. ID. NO. 5) as is noted in WO03/029291, or substantially the same amino acid sequence as is noted in PCT

Application No. US94/02957, and therefore the amino acid sequence of erythropoietin will be used as the native sequence for the mutations disclosed below. However, one of ordinary skill in the art can readily recognize that similar mutations can be used in the modified erythropoietin amino acid sequences of some long acting erythropoietins and that it is preferred that the below mutations not interfere with modifications to the amino acid sequence that are intended to extend the half-life of the long acting erythropoietin. In the mutein nomenclature used throughout herein, the changed amino acid is depicted with the native amino acid's one-letter code first, followed by its position in the erythropoietin molecule, followed by the replacement amino acid one-letter code. For example, "human erythropoietin S100E" or "recombinant tissue protective cytokine S100E" refers to a human erythropoietin molecule in which amino acid 100, a serine, has been changed to glutamic acid. Such muteins useful for the practice of the present invention include but are not limited to long acting erythropoietin with at least one of the following amino acid changes:

I6A, C7A, C7S,
R10I, V11S, L12A, E13A, R14A, R14E, R14Q, Y15A, Y15F, Y15I,
K20E, K20A,
E21A,
N24K, C29S, C29Y, A30N, H32T,
C33S, C33Y, N38K, N83K,
P42N,
P42A, D43A, T44I, K45D, K45A, V46A, N47A, F48I, F48A, Y49A, Y49S, 44-49 deletion,
W51F, W51N, K52A,
Q59N,
E62T,
L67S,
L70A,
D96R, K97A
S100R, S100E, S100A, S100T, G101A, G101I, L102A, R103A, R103E, S104A, S104I,
L105A, T106A, T106I, T107A, T107L, L108K, L108A, L108S,
K116A,
S126A,

T132A,
I133A, T134A,
K140A,
F142I,
R143A,
S146A, N147K, N147A, F148Y, P148A, L149A, R150A, R150E, G151A,
K152A, K152W,
L153A,
K154A,
L155A, G158A,
C160S, C161A, or R162A.

In preferred embodiments, a long acting erythropoietin mutein or a recombinant long acting tissue protective cytokine of the invention comprises one or more of the above substitutions. In other embodiments, long acting erythropoietin muteins or another recombinant long acting tissue protective cytokine of the invention comprises one of the above substitutions or a combination thereof.

In an alternative embodiment, the recombinant long acting tissue protective cytokines, pharmaceutical compositions, use, and treatment methods of the invention comprise one or more of the above substitutions with the proviso that they do not consist of one or more of the following substitutions: I6A, C7A, K20A, P42A, D43A, K45D, K45A, F48A, Y49A, K52A, K49A, S100E, R103A, K116A, T132A, I133A, K140A, N147K, N147A, R150A, R150E, G151A, K152A, K154A, G158A, C161A, or R162A. In a related embodiment of the invention, the recombinant long acting tissue protective cytokines, pharmaceutical compositions, use, and treatment methods of the invention comprise one or more of the above substitutions with the proviso that they do not consist of the following combinations of substitutions: N24K/N38K/N83K or A30N/H32T.

In certain embodiments, more than one of the amino acid changes above can be combined to make a mutein. Examples of such combinations include, but are not limited to: K45D/S100E, A30N/H32T, K45D/R150E, R103E/L108S, K140A/K52A, K140A/K52A/K45A, K97A/K152A, K97A/K152A/K45A, K97A/K152A/K45A/K52A, K97A/K152A/K45A/K52A/K140A, K97A/K152A/K45A/K52A/K140A/K154A,

N24K/N38K/N83K, and N24K/Y15A. In certain embodiments, the recombinant long acting tissue protective cytokine mutein of the invention does not consist of one or more of the above multiple substitutions. In certain embodiments the pharmaceutical compositions of the invention comprising the recombinant long acting tissue protective cytokine mutein of the invention does not consist of one or more of the above multiple substitutions. In certain embodiments the use and treatment methods of the invention which utilize the recombinant long acting tissue protective cytokine mutein of the invention do not consist of one or more of the above multiple substitutions.

Certain modifications or combinations of modifications can affect the flexibility of an erythropoietin muteins and their binding to a receptor, such as the erythropoietin receptor or a secondary receptor to which erythropoietin or an erythropoietin mutein binds. Examples of such modifications or combinations thereof useful in the compositions and methods of the invention, include, but are not limited to, K152W, R14A/Y15A, I6A, C7A, D43A, P42A, F48A, Y49A, T132A, I133A, T134A, N147A, P148A, R150A, G151A, G158A, C161A, and R162A. Corresponding mutations are known to be detrimental in human growth hormone (Wells et al.). In certain embodiments, the recombinant long acting tissue protective cytokine mutein of the invention does not consist of one or more of the above substitutions. In certain embodiments the pharmaceutical compositions of the invention comprising the recombinant long acting tissue protective cytokine mutein of the invention do not consist of one or more of the above substitutions. In certain embodiments the use and treatment methods of the invention which utilize the recombinant long acting tissue protective cytokine mutein of the invention do not consist of one or more of the above substitutions.

A variety of host-expression vector systems may be utilized to produce the recombinant long acting tissue protective cytokines, including long acting erythropoietin mutein molecules of the invention. Such host-expression systems represent vehicles by which the recombinant long acting tissue protective cytokines of interest may be produced and subsequently purified, but also represent cells that may, when transformed or transfected with the appropriate nucleotide coding sequences, exhibit the modified erythropoietin gene product *in situ*. These include, but are not limited to, bacteria, insect, plant, mammalian, including human host systems, such as, but not limited to, insect cell systems infected with recombinant virus expression vectors (*e.g.*, baculovirus) containing

the recombinant long acting tissue protective cytokine product coding sequences; plant cell systems infected with recombinant virus expression vectors (*e.g.*, cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors (*e.g.*, Ti plasmid) containing recombinant long acting tissue protective cytokine coding sequences; or mammalian cell systems, including human cell systems, (*e.g.*, HT1080, COS, CHO, BHK, 293, 3T3) harboring recombinant expression constructs containing promoters derived from the genome of mammalian cells (*e.g.*, metallothionein promoter) or from mammalian viruses (*e.g.*, the adenovirus late promoter; the vaccinia virus 7.5K promoter). The methods for expressing a recombinant protein are well known in the art and are further detailed in PCT/US03/20964, which is incorporated herein by reference in its entirety.

These additional modifications may be used to enhance the tissue protective effect, suppress the bone marrow effect, or alter the physical properties, such as charge, of the long acting tissue protective cytokine. Furthermore, the skilled artisan can readily determine that a long acting tissue protective cytokine useful for the purposes herein may have at least one of the aforementioned modifications, but may have more than one of the above modifications. For example, the skilled artisan would recognize that a carbamylated ARANESP could be further modified by the substitution glutamic acid for a serine at amino acid 100 within the amino acid sequence for ARANESP. Furthermore, with regard to long acting erythropoietins resulting from fusion proteins or diglycosylated and pegylated erythropoietin, skilled artisans would recognize that the modifications can occur to the whole molecule or portions of the molecule during its manufacture. For example, the chemical modification of a long acting erythropoietin that is a fusion protein can occur to the erythropoietin portion of the molecule prior to fusing it to the other protein or to the overall fusion protein. Or in the case of the diglycosylated and pegylated erythropoietin, a skilled artisan may elect to introduce a mutation into C-terminal fragment and chemically modify the N-terminal fragment prior to ligating the two fragments.

Following the manufacture of the long acting tissue protective cytokines of the present invention, one of ordinary skill in the art can verify the tissue protective attributes of the cytokines and the absence of an effect on the bone marrow using well known assays.

For example, the non-erythropoietic affect of a long acting tissue protective cytokine can be verified through the use of an *in vitro* erythroleukemia cell (*e.g.*, TF-1 or UT-7) based assay. In a TF-1 cells are grown in a complete RPMI medium supplemented with 5 ng/ml of GM-CSF and 10% FCS for a day at 37 C in a CO_2 incubator. The cells are then washed in and suspended at a density of 10^6 cells/ml for 16 hours in starvation medium (5% FCS without GM-CSF). A 96 well plate is prepared by: (1) adding 100 μl of sterile water to the outer wells to maintain moisture; (2) adding medium (10% FCS without cells or GM-CSF) alone to 5 wells; and (3) seeding 25,000 cells/well with medium containing 10% FCS and the long acting tissue protective cytokines in remaining wells (five wells per cytokine being tested). If the cells proliferate, the long acting tissue protective cytokine may be erythropoietic. The *in vivo* effect of the compound should then be tested on an *in vivo* assay monitoring an increase of hematocrit due to the long acting tissue protective cytokine. A negative result – non-proliferation of cells in the TF-1 assay *in vitro* assay or no increase in hematocrit within the *in vivo* assay –means that the long acting tissue protective cytokine is nonerythropoietic.

The tissue protective properties of the long acting tissue protective cytokine may be verified using the NMDA toxicity *in vitro* assay outlined in further detail below, as well as the various assays disclosed in PCT/US01/49479 included herein in its entirety by reference. The above assays are provided merely as examples, and other suitable assays to determine the effect of the cytokines on bone marrow and tissue protection are known to those of ordinary skill in the art are contemplated by the present invention as well.

III. Treatment.

In the practice of one aspect of the present invention, a pharmaceutical composition as described above containing a long acting tissue protective cytokine may be administrable to a mammal by any route which provides a sufficient level of a long acting tissue protective cytokine in the vasculature to permit beneficial effects on responsive cells against an injury. "Responsive cell" refers to a mammalian cell whose function or viability may be maintained, promoted, enhanced, regenerated, or in any other way benefited, by exposure to a long acting erythropoietin. Non-limiting examples of such

cells include neuronal, retinal, muscle, heart, lung, liver, kidney, small intestine, adrenal cortex, adrenal medulla, capillary endothelial, testes, ovary, pancreas, skin, bone and endometrial cells. In particular, responsive cells include, without limitation, neuronal cells; retinal cells: photoreceptor (rods and cones), ganglion, bipolar, horizontal, amacrine, and Müller cells; muscle cells; heart cells: myocardium, pace maker, sinoatrial node, sinus node, and junction tissue cells (atrioventricular node and bundle of His); lung cells; liver cells: hepatocytes, stellate, and Kupffer cells; kidney cells: mesangial, renal epithelial, and tubular interstitial cells; small intestine cells: goblet, intestinal gland (crypts) and enteral endocrine cells; adrenal cortex cells: glomerulosa, fasciculate, and reticularis cells; adrenal medulla cells: chromaffin cells; capillary cells: pericyte cells; testes cells: Leydig, Sertoli, and sperm cells and their precursors; ovary cells: Graafian follicle and primordial follicle cells; endometrial cells: endometrial stroma and endometrial cells; pancreas cell: islet of Langerhans, α-cells, β-cells, γ-cells, and δ-cells; skin cells; bone cells: osteoprogenitor, osteoclast and osteoblast cells; as well as the stem and endothelial cells present in the above listed organs. Moreover, such responsive cells and the benefits provided thereto by a long acting erythropoietin or a long acting tissue protective cytokine may be extended to provide protection or enhancement indirectly to other cells that are not directly responsive, or of tissues or organs which contain such non-responsive cells. These other cells, or tissues or organs which benefit indirectly from the enhancement of responsive cells present as part of the cells, tissue or organ as "associated" cells, tissues and organs. Thus, benefits of a long acting erythropoietin or a long acting tissue protective cytokine as described herein may be provided as a result of the presence of a small number or proportion of responsive cells in a tissue or organ, for example, excitable or neuronal tissue present in such tissue, or the Leydig cells of the testis, which makes testosterone. In one aspect, the responsive cell or its associated cells, tissues, or organs are not excitable cells, tissues, or organs, or do not predominantly comprise excitable cells or tissues.

"Injury" refers to the human diseases of the central nervous system or peripheral nervous system which have primarily neurological or psychiatric symptoms, ophthalmic diseases, cardiovascular diseases, cardiopulmonary diseases, respiratory diseases, kidney, urinary and reproductive diseases, bone diseases, skin diseases, gastrointestinal diseases and endocrine and metabolic abnormalities on which the long acting erythropoietin and the long acting tissue protective cytokines of the present invention have a therapeutic

effect. The methods of the invention provide for the local or systemic protection of cells, tissues and organs within a mammalian body or restoration or regeneration of dysfunction resulting from an injury through the administration of a long acting erythropoietin or long acting tissue protective cytokine.

In particular, such conditions and diseases include hypoxic conditions, which adversely affect excitable tissues, such as excitable tissues in the central nervous system tissue, peripheral nervous system tissue, or cardiac tissue or retinal tissue such as, for example, brain, heart, or retina/eye. Any condition which reduces the availability of oxygen to neuronal tissue, resulting in stress, damage, and finally, neuronal cell death, can be treated by the methods of the present invention. Generally referred to as hypoxia and/or ischemia, these conditions arise from or include, but are not limited to stroke, vascular occlusion, prenatal or postnatal oxygen deprivation, suffocation, choking, asthma, near drowning, carbon monoxide poisoning, smoke inhalation, trauma, including surgery and radiotherapy, asphyxia, epilepsy, hypoglycemia, chronic obstructive pulmonary disease, emphysema, adult respiratory distress syndrome, hypotensive shock, septic shock, anaphylactic shock, insulin shock, sickle cell crisis, cardiac arrest, dysrhythmia, nitrogen narcosis, and neurological deficits caused by heart-lung bypass procedures.

The aforementioned long acting tissue protective cytokines are useful generally for the therapeutic or prophylactic treatment of human diseases of the central nervous system or peripheral nervous system which have primarily neurological or psychiatric symptoms, ophthalmic diseases, cardiovascular diseases, cardiopulmonary diseases, respiratory diseases, kidney, urinary and reproductive diseases, bone diseases, skin diseases, gastrointestinal diseases and endocrine and metabolic abnormalities. In particular, such conditions and diseases include hypoxic conditions, which adversely affect excitable tissues, such as excitable tissues in the central nervous system tissue, peripheral nervous system tissue, or cardiac tissue or retinal tissue such as, for example, brain, heart, or retina/eye. Therefore, the invention can be used to treat or prevent damage to excitable tissue resulting from hypoxic conditions in a variety of conditions and circumstances. Non-limiting examples of such conditions and circumstances are provided in the table herein below.

In one embodiment, for example, the specific long acting tissue protective cytokine compositions can be administered to prevent injury or tissue damage resulting from risk of injury or tissue damage during surgical procedures, such as, for example, tumor resection or aneurysm repair. Other pathologies caused by or resulting from hypoglycemia which are treatable by the methods described herein include insulin overdose, also referred to as iatrogenic hyperinsulinemia, insulinoma, growth hormone deficiency, hypocortisolism, drug overdose, and certain tumors.

Other pathologies resulting from excitable neuronal tissue damage include seizure disorders, such as epilepsy, convulsions, or chronic seizure disorders. Other treatable conditions and diseases include diseases such as stroke, multiple sclerosis, hypotension, cardiac arrest, Alzheimer's disease, Parkinson's disease, cerebral palsy, brain or spinal cord trauma, AIDS dementia, age-related loss of cognitive function, memory loss, amyotrophic lateral sclerosis, seizure disorders, alcoholism, retinal ischemia, optic nerve damage resulting from glaucoma, and neuronal loss.

The specific composition and methods of the present invention may be used to treat inflammation resulting from disease conditions or various traumas, such as physically or chemically induced inflammation. Such traumas could include angiitis, chronic bronchitis, pancreatitis, osteomyelitis, rheumatoid arthritis, glomerulonephritis, optic neuritis, temporal arteritis, encephalitis, meningitis, transverse myelitis, dermatomyositis, polymyositis, necrotizing fasciitis, hepatitis, and necrotizing enterocolitis.

The specific compositions and methods of the invention may be used to treat conditions of, and damage to, retinal tissue. Such disorders include, but are not limited to retinal ischemia, macular degeneration, retinal detachment, retinitis pigmentosa, arteriosclerotic retinopathy, hypertensive retinopathy, retinal artery blockage, retinal vein blockage, hypotension, and diabetic retinopathy.

In another embodiment, the methods and principles of the invention may be used to protect or treat injury resulting from radiation damage to excitable tissue. A further

utility of the methods of the present invention is in the treatment of neurotoxin poisoning, such as domoic acid shellfish poisoning, neurolathyrism, and Guam disease, amyotrophic lateral sclerosis, and Parkinson's disease.

As mentioned above, the present invention is also directed to a method for enhancing excitable tissue function in a mammal by peripheral administration of a long acting tissue protective cytokine as described above. Various diseases and conditions are amenable to treatment using this method, and further, this method is useful for enhancing cognitive function in the absence of any condition or disease. These uses of the present invention are describe in further detail below and include enhancement of learning and training in both human and non-human mammals.

Conditions and diseases treatable by the methods of this aspect of the present invention directed to the central nervous system include but are not limited to mood disorders, anxiety disorders, depression, autism, attention deficit hyperactivity disorder, and cognitive dysfunction. These conditions benefit from enhancement of neuronal function. Other disorders treatable in accordance with the teachings of the present invention include sleep disruption, for example, sleep apnea and travel-related disorders; subarachnoid and aneurismal bleeds, hypotensive shock, concussive injury, septic shock, anaphylactic shock, and sequelae of various encephalitides and meningitides, for example, connective tissue disease-related cerebritides such as lupus. Other uses include prevention of or protection from postoperative treatment for embolic or ischemic injury; whole brain irradiation; sickle cell crisis; and eclampsia.

A further group of conditions treatable by the methods of the present invention include mitochondrial dysfunction, of either a hereditary or acquired nature, which are the cause of a variety of neurological diseases typified by neuronal injury and death. For example, Leigh disease (subacute necrotizing encephalopathy) is characterized by progressive visual loss and encephalopathy, due to neuronal drop out, and myopathy. In these cases, defective mitochondrial metabolism fails to supply enough high energy substrates to fuel the metabolism of excitable cells. An erythropoietin receptor activity modulator optimizes failing function in a variety of mitochondrial diseases. As mentioned above, hypoxic conditions adversely affect excitable tissues. The excitable tissues

include, but are not limited to, central nervous system tissue, peripheral nervous system tissue, and heart tissue. In addition to the conditions described above, the methods of the present invention are useful in the treatment of inhalation poisoning such as carbon monoxide and smoke inhalation, severe asthma, adult respiratory distress syndrome, and choking and near drowning. Further conditions which create hypoxic conditions or by other means induce excitable tissue damage include hypoglycemia that may occur in inappropriate dosing of insulin, or with insulin-producing neoplasms (insulinoma).

Various neuropsychologic disorders which are believed to originate from excitable tissue damage are treatable by the instant methods. Chronic disorders in which neuronal damage is involved and for which treatment by the present invention is provided include disorders relating to the central nervous system and/or peripheral nervous system including age-related loss of cognitive function and senile dementia, chronic seizure disorders, Alzheimer's disease, Parkinson's disease, dementia, memory loss, amyotrophic lateral sclerosis, multiple sclerosis, tuberous sclerosis, Wilson's Disease, cerebral and progressive supranuclear palsy, Guam disease, Lewy body dementia, prion diseases, such as spongiform encephalopathies, e.g., Creutzfeldt-Jakob disease, Huntington's disease, myotonic dystrophy, Freidrich's ataxia and other ataxias, as well as Gilles de la Tourette's syndrome, seizure disorders such as epilepsy and chronic seizure disorder, stroke, brain or spinal cord trauma, AIDS dementia, alcoholism, autism, retinal ischemia, glaucoma, autonomic function disorders such as hypertension and sleep disorders, and neuropsychiatric disorders that include, but are not limited to schizophrenia, schizoaffective disorder, attention deficit disorder, dysthymic disorder, major depressive disorder, mania, obsessive-compulsive disorder, psychoactive substance use disorders, anxiety, panic disorder, as well as unipolar and bipolar affective disorders. Additional neuropsychiatric and neurodegenerative disorders include, for example, those listed in the American Psychiatric Association's Diagnostic and Statistical Manual of Mental Disorders (DSM), the most current version of which in incorporated herein by reference in its entirety.

In another embodiment, recombinant chimeric toxin molecules comprising long acting erythropoietin can be used for therapeutic delivery of toxins to treat a proliferative disorder, such as cancer, or viral disorder, such as subacute sclerosing panencephalitis.

Non-limiting examples of such conditions and circumstances are provided in the table herein below.

Cell, tissue or organ	*Dysfunction or pathology*	*Condition or disease*	*Type*
Heart	Ischemia	Coronary artery disease	Acute, chronic Stable, unstable
		Myocardial infarction	
		Angina	
		Congenital heart disease	Valvular Cardiomyopathy
		Prinzmetal angina	
		Cardiac rupture	Aneurysmatic Septal perforation
		Angiitis	
		surgical procedures	Angioplasty
	Arrhythmia	Tachy-, bradyarrhythmia Supraventricular, ventricular Conduction abnormalities	Stable, unstable Hypersensitive carotid sinus node
	Congestive heart failure	Left, right, bi-ventricular	Cardiomyopathies, such as idiopathic familial, infective, metabolic, storage disease, deficiencies, connective tissue disorder, infiltration and granulomas, neurovascular
		Myocarditis	Autoimmune, infective, idiopathic
		Cor pulmonale	
	Blunt and penetrating trauma		
	Toxins	Cocaine	
Vascular	Hypertension	Primary, secondary	
	Decompression sickness		
	Fibromuscular hyperplasia		

Cell, tissue or organ	*Dysfunction or pathology*	*Condition or disease*	*Type*
	Aneurysm	Dissecting, ruptured, enlarging	
Lungs	Obstructive	Asthma Chronic bronchitis, Emphysema and airway obstruction ARDS	
	Ischemic lung disease	Pulmonary embolism, Pulmonary thrombosis, Fat embolism	
	Environmental lung diseases		
	Ischemic lung disease	Pulmonary embolism Pulmonary thrombosis	
	Interstitial lung disease	Idiopathic pulmonary fibrosis	
	Congenital	Cystic fibrosis	
	Cor pulmonale		
	Trauma		
	Pneumonia and pneumonitides	Infectious, parasitic, toxic, traumatic, burn, aspiration	
	Sarcoidosis		
Pancreas	Endocrine	Diabetes mellitus, type I and II	Beta cell failure, dysfunction Diabetic neuropathy
		Other endocrine cell failure of the pancreas	
	Exocrine	Exocrine pancreas failure	Pancreatitis
Bone	Osteopenia	Primary secondary	Hypogonadism immobilisation Postmenopausal Age-related Hyperparathyroidism Hyperthyroidism Calcium, magnesium, phosphorus and/or vitamin D deficiency
	Osteomyelitis		
	Avascular necrosis		

Cell, tissue or organ	*Dysfunction or pathology*	*Condition or disease*	*Type*
	Trauma		
	Paget's disease		
Skin	Alopecia	Areata Totalis	Primary Secondary Male pattern baldness
	Vitiligo	Localized Generalized	Primary Secondary
	Diabetic ulceration		
	Peripheral vascular disease		
	Burn injuries		
Autoimmune disorders	Lupus erythematodes, Sjogren's syndrome, Rheumatoid arthritis, Glomerulonephritis, Angiitis		
	Langerhan's histiocytosis		
Eye	Optic neuritis		
	Blunt and penetrating injuries, Infections, Sareoid, Sickle C disease, Retinal detachment, Temporal arteritis		
	Retinal ischemia, macular degeneration, retinal detachment, retinitis pigmentosa, arteriosclerotic retinopathy, hypertensive retinopathy, retinal artery blockage, retinal vein blockage, hypotension, and diabetic retinopathy.		
Embryonic	Asphyxia		

Cell, tissue or organ	*Dysfunction or pathology*	*Condition or disease*	*Type*
and fetal disorders and pregnancy	Ischemia		
	Eclampsia		
	Ischemic stroke hemorrhagic stroke brain trauma spinal cord trauma epilepsy convulsions chronic seizure disorder		
CNS	Chronic fatigue syndrome, acute and chronic hypoosmolar and hyperosmolar syndromes, AIDS Dementia, Electrocution Asphyxia Multiple sclerosis Alzheimer's disease Parkinson's disease Cerebral palsy Age-related loss of cerebral function Memory loss ALS Seizure disorder Subacute sclerosing panencephalitis		
	Encephalitis	Rabies, Herpes	
	Meningitis		
	Subdural hematoma		
	Nicotine addiction		
	Drug abuse and withdrawal	Cocaine, heroin, crack, marijuana, LSD, PCP, poly-drug abuse, ecstasy, opioids, sedative hypnotics, amphetamines, caffeine, alcohol	

Cell, tissue or organ	*Dysfunction or pathology*	*Condition or disease*	*Type*
	Neuropsychiatric	Obsessive-compulsive disorders, mood disorders, anxiety disorders, depression, autism, attention deficit hyperactivity disorder, cognitive dysfunction.	
	Spinal stenosis, Transverse myelitis, Guillian Barre, Trauma, Nerve root compression, Tumoral compression, Heat stroke tuberous sclerosis, Wilson's Disease, cerebral and progressive supranuclear palsy, Guam disease, Lewy body dementia, Huntington's disease, myotonic dystrophy, Freidrich's ataxia and other ataxias, Gilles de la Tourette's syndrome		
	prion diseases.	spongiform encephalopathies, Creutzfeldt-Jakob disease	
	Cardiopulmonary bypass		
ENT	Tinnitus Meuniere's syndrome Hearing loss		
	Traumatic injury, barotraumas		

Cell, tissue or organ	*Dysfunction or pathology*	*Condition or disease*	*Type*
Kidney	Renal failure	Acute, chronic	Vascular/ischemic, interstitial disease, diabetic kidney disease, nephrotic syndromes, infections
	Henoch-Schonlein Purpura		
	transplant		
Striated muscle	Autoimmune disorders	Myasthenia gravis Dermatomyositis Polymyositis	
	Myopathies	Inherited metabolic, endocrine and toxic	
	Heat stroke		
	Crush injury		
	Rhabdomylosis		
	Mitochondrial disease		
	Infection	Necrotizing fasciitis	
Sexual dysfunction	Central and peripheral	Impotence secondary to medication	
Liver	Hepatitis	Viral, bacterial, parasitic	
	Ischemic disease		
	Cirrhosis, fatty liver		
	Infiltrative/metabolic diseases		
Gastrointestinal	Ischemic bowel disease		
	Inflammatory bowel disease		
	Necrotizing enterocolitis		
Organ transplantation	Treatment of donor and recipient		
Reproductive tract	Infertility	Vascular Autoimmune Uterine abnormalities Implantation disorders	
Endocrine	Glandular hyper-		

Cell, tissue or organ	*Dysfunction or pathology*	*Condition or disease*	*Type*
	and hypofunction		

As mentioned above, these diseases, disorders or conditions are merely illustrative of the range of benefits provided by the long acting tissue protective cytokines of the invention. Accordingly, this invention generally provides therapeutic or prophylactic treatment of the consequences of mechanical trauma or of human diseases, including, but not limited to those disclosed within PCT application No. PCT/US03/21350 and U.S. Provisional Application filed September 29, 2003 entitled Tissue Protective Cytokines for the Treatment and Prevention of Sepsis and the Formation of Adhesions, hereby incorporated by reference. Therapeutic or prophylactic treatment for diseases, disorders or conditions of the CNS and/or peripheral nervous system are preferred. Therapeutic or prophylactic treatment for diseases, disorders or conditions which have a psychiatric component is provided. Therapeutic or prophylactic treatment for diseases, disorders or conditions including but not limited to those having an ophthalmic, cardiovascular, cardiopulmonary, respiratory, kidney, urinary, reproductive, gastrointestinal, endocrine, or metabolic component is provided.

IV. Dosing.

A pharmaceutical composition of long acting erythropoietin or a long acting tissue protective cytokine may be administered systemically to protect or enhance the target cells, tissue or organ. Such administration may be parenterally, via inhalation, or transmucosally, *e.g.*, orally, nasally, rectally, intravaginally, sublingually, submucosally or transdermally. Preferably, administration is parenteral, *e.g.*, via intravenous or intraperitoneal injection, and also including, but is not limited to, intra-arterial, intramuscular, intradermal and subcutaneous administration. One of ordinary skill in the art would understand that the pharmaceutical composition of the present invention may be made of a mixture of the long acting tissue protective cytokines of the present invention as well as erythropoietin or other variants of erythropoieitn.

For other routes of administration, such as by use of a perfusate, injection into an organ, or other local administration, a pharmaceutical composition will be provided which results in similar levels of a long acting tissue protective cytokine as described above. A level of about 15pM –30 nM is preferred.

The pharmaceutical compositions of the invention may comprise a therapeutically effective amount of a compound, and a pharmaceutically acceptable carrier. In a specific embodiment, the term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized foreign pharmacopeia for use in animals, and more particularly in humans. The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the therapeutic is administered. Such pharmaceutical carriers can be sterile liquids, such as saline solutions in water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. A saline solution is a preferred carrier when the pharmaceutical composition is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. Suitable pharmaceutical excipients include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene glycol, water, ethanol and the like. The composition, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. These compositions can take the form of solutions, suspensions, emulsion, tablets, pills, capsules, powders, sustained-release formulations and the like. The composition can be formulated as a suppository, with traditional binders and carriers such as triglycerides. The compounds of the invention can be formulated as neutral or salt forms. Pharmaceutically acceptable salts include those formed with free amino groups such as those derived from hydrochloric, phosphoric, acetic, oxalic, tartaric acids, etc., and those formed with free carboxyl groups such as those derived from sodium, potassium, ammonium, calcium, ferric hydroxides, isopropylamine, triethylamine, 2-ethylamino ethanol, histidine, procaine, etc. Examples of suitable pharmaceutical carriers are described in "Remington's Pharmaceutical Sciences" by E.W. Martin. Such compositions will contain a therapeutically effective amount of the compound, preferably in purified form, together with a suitable amount of carrier so as to provide the form for proper administration to the patient. The formulation should suit the mode of administration.

Formulations for increasing transmucosal adsorption of large molecules such as long acting tissue protective cytokines are also contemplated by the curremnt invention. Pharmaceutical compositions adapted for oral administration may be provided as capsules

or tablets; as powders or granules; as solutions, syrups or suspensions (in aqueous or non-aqueous liquids); as edible foams or whips; or as emulsions. Tablets or hard gelatine capsules may comprise lactose, starch or derivatives thereof, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, stearic acid or salts thereof. Soft gelatine capsules may comprise vegetable oils, waxes, fats, semi-solid, or liquid polyols etc. Solutions and syrups may comprise water, polyols and sugars.

An active agent intended for oral administration may be coated with or admixed with a material that delays disintegration and/or absorption of the active agent in the gastrointestinal tract (*e.g.*, glyceryl monostearate or glyceryl distearate may be used). Thus, the sustained release of an active agent may be achieved over many hours and, if necessary, the active agent can be protected from being degraded within the stomach. Pharmaceutical compositions for oral administration may be formulated to facilitate release of an active agent at a particular gastrointestinal location due to specific pH or enzymatic conditions.

Pharmaceutical compositions adapted for transdermal administration may be provided as discrete patches intended to remain in intimate contact with the epidermis of the recipient for a prolonged period of time. Pharmaceutical compositions adapted for topical administration may be provided as ointments, creams, suspensions, lotions, powders, solutions, pastes, gels, sprays, aerosols or oils. For topical administration to the skin, mouth, eye or other external tissues a topical ointment or cream is preferably used. When formulated in an ointment, the active ingredient may be employed with either a paraffinic or a water-miscible ointment base. Alternatively, the active ingredient may be formulated in a cream with an oil-in-water base or a water-in-oil base. Pharmaceutical compositions adapted for topical administration to the eye include eye drops. In these compositions, the active ingredient can be dissolved or suspended in a suitable carrier, *e.g.*, in an aqueous solvent. Pharmaceutical compositions adapted for topical administration in the mouth include lozenges, pastilles and mouthwashes.

Pharmaceutical compositions adapted for nasal and pulmonary administration may comprise solid carriers such as powders (preferably having a particle size in the range of 20 to 500 microns). Powders can be administered in the manner in which snuff is taken,

i.e., by rapid inhalation through the nose from a container of powder held close to the nose. Alternatively, compositions adopted for nasal administration may comprise liquid carriers, *e.g.*, nasal sprays or nasal drops. Alternatively, inhalation of compounds directly into the lungs may be accomplished by inhalation deeply or installation through a mouthpiece into the oropharynx. These compositions may comprise aqueous or oil solutions of the active ingredient. Compositions for administration by inhalation may be supplied in specially adapted devices including, but not limited to, pressurized aerosols, nebulizers or insufflators, which can be constructed so as to provide predetermined dosages of the active ingredient. In a preferred embodiment, pharmaceutical compositions of the invention are administered into the nasal cavity directly or into the lungs via the nasal cavity or oropharynx.

Pharmaceutical compositions adapted for rectal administration may be provided as suppositories or enemas. Pharmaceutical compositions adapted for vaginal administration may be provided as pessaries, tampons, creams, gels, pastes, foams or spray formulations.

Pharmaceutical compositions adapted for parenteral administration include aqueous and non-aqueous sterile injectable solutions or suspensions, which may contain antioxidants, buffers, bacteriostats and solutes that render the compositions substantially isotonic with the blood of an intended recipient. Other components that may be present in such compositions include water, alcohols, polyols, glycerine and vegetable oils, for example. Compositions adapted for parenteral administration may be presented in unit-dose or multi-dose containers, for example sealed ampules and vials, and may be stored in a freeze-dried (lyophilized) condition requiring only the addition of a sterile liquid carrier, *e.g.*, sterile saline solution for injections, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets. In one embodiment, an autoinjector comprising an injectable solution of an long acting erythropoietin may be provided for emergency use by ambulances, emergency rooms, and battlefield situations, and even for self-administration in a domestic setting, particularly where the possibility of traumatic amputation may occur, such as by imprudent use of a lawn mower. The likelihood that cells and tissues in a severed foot or toe will survive after reattachment may be increased by administering long acting erythropoietin or a long acting tissue protective cytokine to multiple sites in the severed

part as soon as practicable, even before the arrival of medical personnel on site, or arrival of the afflicted individual with severed toe in tow at the emergency room.

In a preferred embodiment, the composition is formulated in accordance with routine procedures as a pharmaceutical composition adapted for intravenous administration to human beings. Typically, compositions for intravenous administration are solutions in sterile isotonic aqueous buffer. Where necessary, the composition may also include a solubilizing agent and a local anesthetic such as lidocaine to ease pain at the site of the injection. Generally, the ingredients are supplied either separately or mixed together in unit dosage form, for example, as a dry lyophilized powder or water-free concentrate in a hermetically-sealed container such as an ampule or sachette indicating the quantity of active agent. Where the composition is to be administered by infusion, it can be dispensed with an infusion bottle containing sterile pharmaceutical grade water or saline. Where the composition is administered by injection, an ampule of sterile saline can be provided so that the ingredients may be mixed prior to administration.

Suppositories generally contain active ingredient in the range of 0.5% to 10% by weight; oral formulations preferably contain 10% to 95% active ingredient.

A perfusate composition may be provided for use in transplanted organ baths, for in situ perfusion, or for administration to the vasculature of an organ donor prior to organ harvesting. Such pharmaceutical compositions may comprise levels of long acting erythropoietin, long acting tissue protective cytokines, or a form of either long acting erythropoietin or long acting tissue protective cytokines not suitable for acute or chronic, local or systemic administration to an individual, but will serve the functions intended herein in a cadaver, organ bath, organ perfusate, or in situ perfusate prior to removing or reducing the levels of the long acting erythropoietin contained therein before exposing or returning the treated organ or tissue to regular circulation. The long acting erythropoietin for this aspect of the invention may be any long acting erythropoietin, such as naturally-occurring forms such as human erythropoietin, or any of long acting tissue protective cytokines hereinabove described, carbamylated ARANESP, as a non-limiting example.

The invention also provides a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the pharmaceutical compositions of the invention. Optionally associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration.

In another embodiment, for example, a long acting tissue protective cytokine can be delivered in a controlled-release system. For example, the polypeptide may be administered using intravenous infusion, an implantable osmotic pump, a transdermal patch, liposomes, or other modes of administration. In one embodiment, a pump may be used (see Langer, *supra;* Sefton, 1987, CRC Crit. Ref. Biomed. Eng. 14:201; Buchwald *et al.*, 1980, Surgery 88:507; Saudek *et al.*, 1989, N. Engl. J. Med. 321:574). In another embodiment, the compound can be delivered in a vesicle, in particular a liposome (*see* Langer, *Science* 249:1527-1533 (1990); Treat *et al.*, in Liposomes in the Therapy of Infectious Disease and Cancer, Lopez-Berestein and Fidler (eds.), Liss, New York, pp. 353-365 (1989); WO 91/04014; U.S. Patent No. 4,704,355; Lopez-Berestein, *ibid.*, pp. 317-327; *see* generally *ibid.*). In another embodiment, polymeric materials can be used (see Medical Applications of Controlled Release, Langer and Wise (eds.), CRC Press: Boca Raton, Florida, 1974; Controlled Drug Bioavailability, Drug Product Design and Performance, Smolen and Ball (eds.), Wiley: New York (1984); Ranger and Peppas, J. Macromol. Sci. Rev. Macromol. Chem. 23:61, 1953; see also Levy *et al.*, 1985, Science 228:190; During *et al.*, 1989, Ann. Neurol. 25:351; Howard *et al.*, 1989, J. Neurosurg. 71:105).

In yet another embodiment, a controlled release system can be placed in proximity of the therapeutic target, *i.e.*, the target cells, tissue or organ, thus requiring only a fraction of the systemic dose (see, *e.g.*, Goodson, pp. 115-138 in Medical Applications of Controlled Release, vol. 2, *supra*, 1984). Other controlled release systems are discussed in the review by Langer (1990, Science 249:1527-1533).

In another embodiment, a long acting tissue protective cytokine, as properly formulated, can be administered by nasal, oral, rectal, vaginal, or sublingual administration.

In a specific embodiment, it may be desirable to administer long acting erythropoietin and/or the long acting tissue protective cytokines of the invention locally to the area in need of treatment; this may be achieved by, for example, and not by way of limitation, local infusion during surgery, topical application, *e.g.*, in conjunction with a wound dressing after surgery, by injection, by means of a catheter, by means of a suppository, or by means of an implant, said implant being of a porous, non-porous, or gelatinous material, including membranes, such as silastic membranes, or fibers. A non-limiting example of such an embodiment would be a coronary stent coated with a long acting tissue protective cytokine of the present invention.

Selection of the preferred effective dose will be readily determinable by a skilled artisan based upon considering several factors, which will be known to one of ordinary skill in the art. Such factors include the particular form of long acting erythropoietin or the long acting tissue protective cytokine, and its pharmacokinetic parameters such as bioavailability, metabolism, half-life, etc., which will have been established during the usual development procedures typically employed in obtaining regulatory approval for a pharmaceutical compound. Further factors in considering the dose include the condition or disease to be treated or the benefit to be achieved in a normal individual, the body mass of the patient, the route of administration, whether administration is acute or chronic, concomitant medications, and other factors well known to affect the efficacy of administered pharmaceutical agents. Thus the precise dosage should be decided according to the judgment of the practitioner and each patient's circumstances, *e.g.*, depending upon the condition and the immune status of the individual patient, and according to standard clinical techniques.

In another aspect of the present invention, a pharmaceutical composition according to the present invention may include a long acting erythropoietin and/or long acting tissue protective cytokine in a formulation with at least one small molecule that exhibits tissue protective functionality. Suitable small molecules include, but are not limited to, steroids

(*e.g.*, lazaroids and glucocorticoids), antioxidants (*e.g.*, coenzyme Q_{10}, alpha lipoic acid, and NADH), anticatabolic enzymes (*e.g.*, glutathione peroxidase, superoxide dimutase, catalase, synthetic catalytic scavengers, as well as mimetics), indole derivatives (*e.g.*, indoleamines, carbazoles, and carbolines), nitric acid neutralizing agents, adenosine / adenosine agonists, phytochemicals (flavanoids), herbal extracts (ginko biloba and turmeric), vitamins (vitamins A, E, and C), oxidase electron acceptor inhibitors (*e.g.*, xanthine oxidase electron inhibitors), minerals (*e.g.*, copper, zinc, and magnesium), non-steriodal anti-inflammatory drugs (*e.g.*, aspirin, naproxen, and ibuprofen), and combinations thereof. Additionally agents including, but not limited to, anti-inflammatory agents (*e.g.*, corticosteroids, prednisone and hydrocortisone), glucocorticoids, steroids, non-steriodal anti-inflammatory drugs (*e.g.*, aspirin, ibuprofen, diclofenac, and COX-2 inhibitors), beta-agonists, anticholinergic agents and methyl xanthines), immunomodulatory agents (*e.g.*, small organic molecules, a T cell receptor modulators, cytokine receptor modulators, T-cell depleting agents, cytokine antagonists, monokine antagonists, lymphocyte inhibitors, or anti-cancer agents), gold injections, sulphasalazine, penicillamine, anti-angiogenic agents (*e.g.*, angiostatin, TNF-α antagonists (*e.g.*, anti-TNFα antibodies), and endostatin), dapsone, psoralens (*e.g.*, methoxalen and trioxsalen), anti-malarial agents (*e.g.*, hydroxychloroquine), anti-viral agents, and antibiotics (*e.g.*, erythromycin and penicillin) may be used in conjunction with the current pharmaceutical compositions.

In another aspect of the invention, a perfusate or perfusion solution is provided for perfusion and storage of organs for transplant, the perfusion solution includes an amount of long acting erythropoietin or a long acting tissue protective cytokine effective to protect responsive cells and associated cells, tissues or organs. Transplant includes but is not limited to xenotransplantation, where an organ (including cells, tissue or other bodily part) is harvested from one donor and transplanted into a different recipient; and autotransplant, where the organ is taken from one part of a body and replaced at another, including bench surgical procedures, in which an organ may be removed, and while *ex vivo*, resected, repaired, or otherwise manipulated, such as for tumor removal, and then returned to the original location. In one embodiment, the perfusion solution is the University of Wisconsin (UW) solution (U.S. Patent No. 4,798,824) which contains from about 1 to about 25 U/ml long acting erythropoietin, 5% hydroxyethyl starch (having a molecular

weight of from about 200,000 to about 300,000 and substantially free of ethylene glycol, ethylene chlorohydrin, sodium chloride and acetone); 25mM KH_2PO_4; 3mM glutathione; 5mM adenosine; 10mM glucose; 10mM HEPES buffer; 5mM magnesium gluconate; 1.5mM $CaCl_2$; 105mM sodium gluconate; 200,000 units penicillin; 40 units insulin; 16mg dexamethasone; 12mg Phenol Red; and has a pH of 7.4-7.5 and an osmolality of about 320 mOsm/l. The solution is used to maintain cadaveric kidneys and pancreases prior to transplant. Using the solution, preservation can be extended beyond the 30-hour limit recommended for cadaveric kidney preservation. This particular perfusate is merely illustrative of a number of such solutions that can be adapted for the present use by inclusion of an effective amount of long acting erythropoietin and/or a long acting tissue protective cytokine. In a further embodiment, the perfusate solution contains from about 1 to about 500 ng/ml long acting erythropoietin, or from about 40 to about 320 ng/ml long acting erythropoietin. As mentioned above, any form of long acting erythropoietin or long acting tissue protective cytokines can be used in this aspect of the invention.

While the preferred recipient of a long acting tissue protective cytokine for the purposes herein throughout is a human, the methods herein apply equally to other mammals, particularly domesticated animals, livestock, companion, and zoo animals. However, the invention is not so limiting and the benefits can be applied to any mammal.

In further aspects of the *ex-vivo* invention, long acting erythropoietin and any long acting tissue protective cytokine such as but not limited to the ones described above may be employed.

In another aspect of the invention, methods and compositions for enhancing the viability of cells, tissues or organs which are not isolated from the vasculature by an endothelial cell barrier are provided by exposing the cells, tissue or organs directly to a pharmaceutical composition comprising long acting erythropoietin or a long acting tissue protective cytokine, or administering or contacting a pharmaceutical composition containing long acting erythropoietin or a long acting tissue protective cytokine to the vasculature of the tissue or organ. Enhanced activity of responsive cells in the treated tissue or organ is responsible for the positive effects exerted.

As described above, the invention is based, in part, on the discovery that long acting erythropoietin molecules can be transported from the luminal surface to the basement membrane surface of endothelial cells of the capillaries of organs with endothelial cell tight junctions, including, for example, the brain, retina, and testis. Thus, responsive cells across the barrier are susceptible targets for the beneficial effects of long acting erythropoietin or long acting tissue protective cytokines, and others cell types or tissues or organs that contain and depend in whole or in part on responsive cells therein are targets for the methods of the invention. While not wishing to be bound by any particular theory, after transcytosis of long acting erythropoietin or the long acting tissue protective cytokine, long acting erythropoietin or the long acting tissue protective cytokine can interact with an erythropoietin receptor on a responsive cell, for example, neuronal, retinal, muscle, heart, lung, liver, kidney, small intestine, adrenal cortex, adrenal medulla, capillary endothelial, testes, ovary, or endometrial cell, and receptor binding can initiate a signal transduction cascade resulting in the activation of a gene expression program within the responsive cell or tissue, resulting in the protection of the cell or tissue, or organ, from damage, such as by toxins, chemotherapeutic agents, radiation therapy, hypoxia, *etc.* Thus, methods for protecting responsive cell-containing tissue from injury or hypoxic stress, and enhancing the function of such tissue are described in detail herein below.

In the practice of one embodiment of the invention, a mammalian patient is undergoing systemic chemotherapy for cancer treatment, including radiation therapy, which commonly has adverse effects such as nerve, lung, heart, ovarian or testicular damage. Administration of a pharmaceutical composition comprising a long acting erythropoietin and/or a long acting tissue protective cytokine as described above is performed prior to and during chemotherapy and/or radiation therapy, to protect various tissues and organs from damage by the chemotherapeutic agent, such as to protect the testes. Treatment may be continued until circulating levels of the chemotherapeutic agent have fallen below a level of potential danger to the mammalian body.

In the practice of another embodiment of the invention, various organs are planned to be harvested from a victim of an automobile accident for transplant into a number of recipients, some of which required transport for an extended distance and period of time. Prior to organ harvesting, the victim is infused with a pharmaceutical composition

comprising long acting erythropoietins and /or long acting tissue protective cytokines as described herein. Harvested organs for shipment are perfused with a perfusate containing long acting erythropoietins and/or long acting tissue protective cytokines as described herein, and stored in a bath comprising long acting erythropoietins and/or long acting tissue protective cytokines. Certain organs are continuously perfused with a pulsatile perfusion device, utilizing a perfusate containing long acting erythropoietins and/or long acting tissue protective cytokines in accordance with the present invention. Minimal deterioration of organ function occurrs during the transport and upon implant and reperfusion of the organs *in situ*.

In another embodiment of the present invention, a participant in a hazardous activity, one could take a dose of a pharmaceutical composition containing a long acting erythropoietin and/or a long acting tissue protective cytokine sufficient to either prevent (i.e. delaying the onset of, inhibiting, or stopping), protect against, or mitigate the damage resulting from an injury to a responsive cell, tissue, or organ. In particular, this method of treatment may have application in various professions susceptible to injury such as, but not limited to, professional athletes (divers, race car drivers, football players, etc.), military personnel (soldiers, paratroopers), emergency personnel (police, fire, EMS, and disaster relief personnel), stuntmen, and construction workers. Additionally, the prophylactic use of tissue protective cytokines is contemplated in such recreational endeavors including, but not limited to, rock climbing, rappelling, sky diving, racing, bicycling, football, rugby, baseball, and diving that pose a risk of injury. Furthermore, given the extended half-life of a long acting tissue protective cytokine it may provide extended protection against injury to responsive cells, tissues or organs where an individual engages in a hazardous activity for a prolonged period without continued access to a prophylactic supply of tissue protective cytokines, for example mountain climbing or combat.

In another embodiment of the invention, a surgical procedure to repair a heart valve requires temporary cardioplegia and arterial occlusion. Prior to surgery, the patient is infused with a long acting tissue protective cytokine, 4 μg of carbamylated long acting erythropoietin per kg body weight. Such treatment prevents hypoxic ischemic cellular damage, particularly after reperfusion. Additionally, the pharmaceutical compositions of

the present invention may be used prophylactically to prepare an individual for surgery in an effort to limit the trauma associated with the surgical procedure or aide in the recovery of the individual from the surgical procedure. Although the present method of treatment using pharmaceutical compositions containing long acting erythropoietins and/or long acting tissue protective cytokines provides a prophylactic use for surgical procedures, it may be particularly useful in procedures that induce temporary ischemic events including, but not limited to, bypass procedures (coronary bypass), angioplasty procedures, amputations, and transplantations, as well as, those performed directly upon responsive cells, tissues, or organs such as brain and spinal cord surgery, and open heart procedures. Such procedures may involve the use of cardiopulmonary (heart lung) bypass.

In another embodiment of the invention, in any surgical procedure, such as in cardiopulmonary bypass surgery, a long acting erythropoietin or a long acting tissue protective cytokine of the invention can be used. In one embodiment, administration of a pharmaceutical composition comprising long acting erythropoietins and/or long acting tissue protective cytokines as described above is performed prior to, during, and/or following the bypass procedure, to protect the function of brain, heart, and other organs.

In the foregoing examples in which a long acting erythropoietin and/or a long acting tissue protective cytokine of the invention is used for *ex-vivo* applications, or for *in vivo* applications to treat responsive cells such as neuronal tissue, retinal tissue, heart, lung, liver, kidney, small intestine, adrenal cortex, adrenal medulla, capillary endothelial, testes, ovary, or endometrial cells or tissue, the invention provides a pharmaceutical composition in dosage unit form adapted for protection or enhancement of responsive cells, tissues or organs distal to the vasculature which comprises an amount within the range from about 1 pg to 5 mg, 500 pg to 5mg, 1 ng to 5 mg, 500 ng to 5 mg, 1 μg to 5 mg, 500 μg to 5 mg, or 1 mg to 5 mg of a long acting tissue protective cytokine, and a pharmaceutically acceptable carrier. In a preferred embodiment, the amount of long acting tissue protective cytokine is within the range from about 1 pg to 1 mg. In a preferred embodiment, the formulation contains long acting tissue protective cytokines that are non-erythropoietic.

In a further aspect of the invention, administration of long acting tissue protective cytokines is used to restore cognitive function in animals having undergone brain trauma. After a delay of either 5 days or 30 days, administration of long acting erythropoietin is still able to restore function as compared to placebo-treated animals, indicating the ability of an long acting erythropoietin to regenerate or restore brain activity. Thus, the invention is also directed to the use of long acting erythropoietin and/or long acting tissue protective cytokines for the preparation of a pharmaceutical composition for the treatment of brain trauma and other cognitive dysfunctions, including treatment well after the injury (e.g. three days, five days, a week, a month, or longer). The invention is also directed to a method for the treatment of cognitive dysfunction following injury by administering an effective amount of long acting erythropoietin and/or long acting tissue protective cytokines. Any long acting erythropoietin and/or long acting tissue protective cytokine as described herein may be used for this aspect of the invention.

Furthermore, this restorative aspect of the invention is directed to the use of any long acting erythropoietins and/or long acting tissue protective cytokines herein for the preparation of a pharmaceutical composition for the restoration of cellular, tissue or organ dysfunction, wherein treatment is initiated after, and well after, the initial insult responsible for the dysfunction. Moreover, treatment using long acting erythropoietin and/or long acting tissue protective cytokines of the invention can span the course of the disease or condition during the acute phase as well as a chronic phase.

A long acting erythropoietin of the invention may be administered systemically at a dosage between about 1 μg and about 100 μg /kg body weight, preferably about 5 -50 μg /kg-body weight, most preferably about 10-30 μg /kg-body weight, per administration. This effective dose should be sufficient to achieve serum levels of long acting erythropoietin greater than about 10,000, 15,000, or 20,000 mU/ml (80, 120, or 160 ng/ml) of serum after long acting erythropoietin administration. Such serum levels may be achieved at about 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 hours post-administration. Such dosages may be repeated as necessary. For example, administration may be repeated daily, as long as clinically necessary, or after an appropriate interval, *e.g.*, every 1 to 12 weeks, preferably, every 1 to 3 weeks. In one embodiment, the effective amount of long acting erythropoietin and a pharmaceutically acceptable carrier may be packaged in a single dose

vial or other container. In another embodiment, the long acting tissue protective cytokines, which are capable of exerting the activities described herein but not causing an increase in hemoglobin concentration or hematocrit, are used. Such long acting tissue protective cytokines are preferred in instances wherein the methods of the present invention are intended to be provided chronically. In another embodiment, a long acting erythropoietin is given at a dose greater than that necessary to maximally stimulate erythropoiesis. As noted above, a long acting tissue protective cytokine of the invention does not necessarily have erythropoietic activity, and therefore the above dosages expressed in hematopoietic units are merely exemplary for long acting erythropoietins that are erythropoietic; hereinabove weight equivalents for dosages are provided which are applicable to long acting tissue protective cytokines.

V. Transcytosis.

The present invention is further directed to a method for facilitating the transport of a molecule across an endothelial cell barrier in a mammal by administering a composition which comprises the particular molecule in association with a long acting erythropoietin or long acting tissue protective cytokine as described hereinabove. As described above, tight junctions between endothelial cells in certain organs in the body create a barrier to the entry of certain molecules. For treatment of various conditions within the barriered organ, means for facilitating passage of pharmaceutical agents is desired. Long acting erythropoietin or long acting tissue protective cytokines of the invention are useful as carriers for delivering other molecules across the blood-brain and other similar barriers. A composition comprising a molecule desirous of crossing the barrier with long acting erythropoietin or a long acting tissue protective cytokine is prepared and peripheral administration of the composition results in the transcytosis of the composition across the barrier. The association between the molecule to be transported across the barrier and the long acting erythropoietin or long acting tissue protective cytokine may be a labile covalent bond, in which case the molecule is released from association with the long acting erythropoietin or long acting tissue protective cytokine after crossing the barrier. If the desired pharmacological activity of the molecule is maintained or unaffected by association with long acting erythropoietin and or long acting tissue protective cytokine, such a complex can be administered.

The skilled artisan will be aware of various means for associating molecules with long acting erythropoietin or a long acting tissue protective cytokine of the invention and the other agents described above, by covalent, non-covalent, and other means. Furthermore, evaluation of the efficacy of the composition can be readily determined in an experimental system. Association of molecules with long acting erythropoietin or a long acting tissue protective cytokine may be achieved by any number of means, including labile, covalent binding, cross-linking, etc. Biotin/avidin interactions may be employed; for example, a biotinylated long acting erythropoietin of the invention may be complexed with a labile conjugate of avidin and a molecule desirably transported. As mentioned above, a hybrid molecule may be prepared by recombinant or synthetic means, for example, a fusion or chimeric polypeptide which includes both the domain of the molecule with desired pharmacological activity and the domain responsible for long acting erythropoietin receptor activity modulation. Protease cleavage sites may be included in the molecule.

A molecule may be conjugated to long acting erythropoietin or a long acting tissue protective cytokine of the invention through a polyfunctional molecule, *i.e.*, a polyfunctional crosslinker. As used herein, the term "polyfunctional molecule" encompasses molecules having one functional group that can react more than one time in succession, such as formaldehyde, as well as molecules with more than one reactive group. As used herein, the term "reactive group" refers to a functional group on the crosslinker that reacts with a functional group on a molecule (*e.g.*, peptide, protein, carbohydrate, nucleic acid, particularly a hormone, antibiotic, or anti-cancer agent to be delivered across an endothelial cell barrier) so as to form a covalent bond between the cross-linker and that molecule. The term "functional group" retains its standard meaning in organic chemistry. The polyfunctional molecules that can be used are preferably biocompatible linkers, *i.e.*, they are noncarcinogenic, nontoxic, and substantially non-immunogenic *in vivo*. Polyfunctional cross-linkers such as those known in the art and described herein can be readily tested in animal models to determine their biocompatibility. The polyfunctional molecule is preferably bifunctional. As used herein, the term "bifunctional molecule" refers to a molecule with two reactive groups. The bifunctional molecule may be heterobifunctional or homobifunctional. A

heterobifunctional cross-linker allows for vectorial conjugation. It is particularly preferred for the polyfunctional molecule to be sufficiently soluble in water for the cross-linking reactions to occur in aqueous solutions such as in aqueous solutions buffered at pH 6 to 8, and for the resulting conjugate to remain water soluble for more effective bio-distribution. Typically, the polyfunctional molecule covalently bonds with an amino or a sulfhydryl functional group. However, polyfunctional molecules reactive with other functional groups, such as carboxylic acids or hydroxyl groups, are contemplated in the present invention.

The homobifunctional molecules have at least two reactive functional groups, which are the same. The reactive functional groups on a homobifunctional molecule include, for example, aldehyde groups and active ester groups. Homobifunctional molecules having aldehyde groups include, for example, glutaraldehyde and subaraldehyde. The use of glutaraldehyde as a cross-linking agent was disclosed by Poznansky et al., Science 223, 1304-1306 (1984). Homobifunctional molecules having at least two active ester units include esters of dicarboxylic acids and N-hydroxysuccinimide. Some examples of such N-succinimidyl esters include disuccinimidyl suberate and dithio-bis-(succinimidyl propionate), and their soluble bis-sulfonic acid and bis-sulfonate salts such as their sodium and potassium salts. These homobifunctional reagents are available from Pierce, Rockford, Illinois.

The heterobifunctional molecules have at least two different reactive groups. The reactive groups react with different functional groups, *e.g.*, present on the long acting erythropoietin and the molecule. These two different functional groups that react with the reactive group on the heterobifunctional cross-linker are usually an amino group, *e.g.*, the epsilon amino group of lysine; a sulfhydryl group, *e.g.*, the thiol group of cysteine; a carboxylic acid, *e.g.*, the carboxylate on aspartic acid; or a hydroxyl group, *e.g.*, the hydroxyl group on serine.

Of course, certain of the various long acting tissue protective cytokines of the invention, and long acting erythropoietin, may not have suitable reactive groups available for use with certain cross-linking agent; however, one of skill in the art will be amply

aware of the choice of cross-linking agents based on the available groups for cross-linking in long acting erythropoietin or long acting tissue protective cytokines of the invention.

When a reactive group of a heterobifunctional molecule forms a covalent bond with an amino group, the covalent bond will usually be an amido or imido bond. The reactive group that forms a covalent bond with an amino group may, for example, be an activated carboxylate group, a halocarbonyl group, or an ester group. The preferred halocarbonyl group is a chlorocarbonyl group. The ester groups are preferably reactive ester groups such as, for example, an N-hydroxy-succinimide ester group.

The other functional group typically is either a thiol group, a group capable of being converted into a thiol group, or a group that forms a covalent bond with a thiol group. The covalent bond will usually be a thioether bond or a disulfide. The reactive group that forms a covalent bond with a thiol group may, for example, be a double bond that reacts with thiol groups or an activated disulfide. A reactive group containing a double bond capable of reacting with a thiol group is the maleimido group, although others, such as acrylonitrile, are also possible. A reactive disulfide group may, for example, be a 2-pyridyldithio group or a 5, 5'-dithio-bis-(2-nitrobenzoic acid) group. Some examples of heterobifunctional reagents containing reactive disulfide bonds include N-succinimidyl 3-(2-pyridyl-dithio) propionate (Carlsson, et al., 1978, Biochem J., 173:723-737), sodium S-4-succinimidyloxycarbonyl-alpha-methylbenzylthiosulfate, and 4-succinimidyloxycarbonyl-alpha-methyl-(2-pyridyldithio)toluene. N-succinimidyl 3-(2-pyridyldithio) propionate is preferred. Some examples of heterobifunctional reagents comprising reactive groups having a double bond that reacts with a thiol group include succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate and succinimidyl m-maleimidobenzoate.

Other heterobifunctional molecules include succinimidyl 3-(maleimido) propionate, sulfosuccinimidyl 4-(p-maleimido-phenyl) butyrate, sulfosuccinimidyl 4-(N-maleimidomethyl- cyclohexane)-1-carboxylate, maleimidobenzoyl-N-hydroxy-succinimide ester. The sodium sulfonate salt of succinimidyl m-maleimidobenzoate is preferred. Many of the above-mentioned heterobifunctional reagents and their sulfonate salts are available from Pierce Chemical Co., Rockford, Illinois USA.

The need for the above-described conjugated to be reversible or labile may be readily determined by the skilled artisan. A conjugate may be tested in vitro for both the long acting erythropoietin, and for the desirable pharmacological activity. If the conjugate retains both properties, its suitability may then be tested *in vivo*. If the conjugated molecule requires separation from long acting erythropoietin or the long acting tissue protective cytokine for activity, a labile bond or reversible association with long acting erythropoietin or the long acting tissue protective cytokine will be preferable. The lability characteristics may also be tested using standard in vitro procedures before *in vivo* testing.

Additional information regarding how to make and use these as well as other polyfunctional reagents may be obtained from the following publications or others available in the art:

Carlsson, J. et al., 1978, Biochem. J. 173:723-737.
Cumber, J.A. et al., 1985, Methods in Enzymology 112:207-224.
Jue, R. et al., 1978, Biochem 17:5399-5405.
Sun, T.T. et al., 1974, Biochem. 13:2334-2340.
Blattler, W.A. et al., 1985, Biochem. 24:1517-152.
Liu, F.T. et al., 1979, Biochem. 18:690-697.
Youle, R.J. and Neville, D.M. Jr., 1980, Proc. Natl. Acad. Sci. U.S.A. 77:5483-5486.
Lerner, R.A. et al., 1981, Proc. Natl. Acad. Sci. U.S.A. 78:3403-3407.
Jung, S.M. and Moroi, M., 1983, Biochem. Biophys. Acta 761:162.
Caulfield, M.P. et al., 1984, Biochem. 81:7772-7776.
Staros, J.V., 1982, Biochem. 21:3950-3955.
Yoshitake, S. et al., 1979, Eur. J. Biochem. 101:395-399.
Yoshitake, S. et al., 1982, J. Biochem. 92:1413-1424.
Pilch, P.F. and Czech, M.P., 1979, J. Biol. Chem. 254:3375-3381.
Novick, D. et al., 1987, J. Biol. Chem. 262:8483-8487.
Lomant, A.J. and Fairbanks, G., 1976, J. Mol. Biol. 104:243-261.
Hamada, H. and Tsuruo, T., 1987, Anal. Biochem. 160:483-488.
Hashida, S. et al., 1984, J. Applied Biochem. 6:56-63.

Additionally, methods of cross-linking are reviewed by Means and Feeney, 1990, Bioconjugate Chem. 1:2-12.

Barriers which are crossed by the above-described methods and compositions of the present invention include but are not limited to the blood-brain barrier, the blood-eye barrier, the blood-testes barrier, the blood-ovary barrier, and the blood-uterus barrier.

Candidate molecules for transport across an endothelial cell barrier include, for example, hormones, such as growth hormone, neurotrophic factors, antibiotics, antivirals, or antifungals such as those normally excluded from the brain and other barriered organs, peptide radiopharmaceuticals, antisense drugs, antibodies and antivirals against biologically-active agents, pharmaceuticals, and anti-cancer agents. Non-limiting examples of such molecules include hormones such as growth hormone, nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF), basic fibroblast growth factor (bFGF), transforming growth factor β1 (TGFβ1), transforming growth factor β2 (TGFβ2), transforming growth factor β3 (TGFβ3), interleukin 1, interleukin 2, interleukin 3, and interleukin 6, AZT, antibodies against tumor necrosis factor, and immunosuppressive agents such as cyclosporin. Additionally, dyes or markers may be attached to long acting erythropoietin or one of the long acting tissue protective cytokines of the present invention in order to visualize cells, tissues, or organs within the brain and other barriered organs for diagnostic purposes. As an example, a marker used to visualize plaque within the brain could be attached to long acting erythropoietin or a long acting tissue protective cytokine in order to determine the progression of Alzheimer's disease within a patient.

The present invention is also directed to a composition comprising a molecule to be transported via transcytosis across an endothelial cell tight junction barrier and an long acting erythropoietin or long acting tissue protective cytokine as described above. The invention is further directed to the use of a conjugate between a molecule and a long acting erythropoietin or a long acting tissue protective cytokine as described above for the preparation of a pharmaceutical composition for the delivery of the molecule across a barrier as described above.

Various animal models and in-vitro tests of neuroprotection and transcytosis are provided in PCT/US01/49479 to demonstrate the effectiveness of the long acting tissue protective cytokines of the invention. For transcytosis, model proteins conjugated to the long acting erythropoietins of the invention are evaluated for transport into the brain following parenteral administration. These tests in in-vitro models and animal models are predictive of the efficacy of the present compounds in other mammalian species including humans.

The present invention may be better understood by reference to the following non-limiting Examples, which are provided as exemplary of the invention. The following examples are presented in order to more fully illustrate the preferred embodiments of the invention. They should in no way be construed, however, as limiting the broad scope of the invention.

Example 1

Production of Carbamylated NESP.

Carbamylated ARANESP was synthesized from ARANESP in accordance with the procedure set forth in Leist et al., Derivatives of Erythropoietin that are Tissue Protective but Not Erythropoietic, SCIENCE 305:239-242 (2004). Briefly, one volume of ARANESP (~1 mg/ml) was mixed with one volume of 1 M Na-borate, (pH ~ 8.8) and recrystallized KOCN was added to a final concentration of 1 M. The mixture was incubated at 37 °C for 24 h. Samples were immediately dialysed against milli-Q water, and subsequently against 20 mM sodium citrate in 0.1 M NaCl, pH 6.0. After dialysis, the samples were concentrated to ~2 mg/ml by Centricon (Amicon) and the protein content determined by the BCA method of Pierce (Rockford, IL). The product was then analyzed by *in vitro* tests for erythropoiesis (UT-7 cells) and neuroprotection (Primary Hippocampal cells). Two batches of fully carbamylated ARANESP, *i.e.* all of the lysines within ARANESP were converted to homocitruline, were generated using this procedure.

A. EPO BIO-ASSAY UT-7 CELL PROLIFERATION

UT-7 is an Epo-dependent leukaemia cell line used for the determination of the erythroid effect of long acting tissue protective cytokine such as carbamylated ARANESP. The UT-7 cells (Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Cat. No. ACC 363) were normally grown in the presence of 10% FBS and 5 ng/ml Epo. The proliferation/survival (= viability increase) response of the cells exposed to Epo is mediated by the classical peripheral-type Epo receptor. The proliferation response is a quantitative measure of and correlates with the capacity of Epo-variants to stimulate the classical Epo receptor.

Methods for UT-7 cell Viability assay

The human leukemia cell line UT7 was made Epo dependent, and the proliferative response to added Epo/long acting tissue protective cytokines was used as a measurement for their biological activity. On day one of the assay, the cells were transferred to fresh complete RPMI 1640 media with 10% serum containing Epo (5 ng/ml) (10% donor calf serum, 4mM L-glutamine, supplemented with 5ng/ml of rhuEPO). The cells were grown in the 75cm^2 flasks with 20 ml of culture/flask. On day two of the assay the cells were transferred from the flask(s) into a 50-m1 conical tube and centrifuge at 1,000 rpm for 5 minutes at room temperature. The old media was discard and the cells were washed two times with 10 ml of starvation media (3% donor calf serum, 4mM L-glutamine). The cells were re-suspended in starvation media, using up and down pipet action to obtain a single cell suspension. To determine the cell density, the re-suspended cells were diluted with starvation media to a density of 4 x 10^5 cells/ml with a total culture volume of 10 ml and placed in a 25 cm^2 flask. The mixture was incubated for 4 h in a humidified incubator with 5% CO_2 at 37°C. During the last hour of incubation, a 96 well plate was prepared. At the end of the 4-hour incubation, the cell cultures were removed from the incubator, and the cells were transferred from a flask to a 50-ml conical tube. The contents were mixed by hand to keep the cells suspended. 50 m1 of starvation media was added as the media blank without cells. Five wells were the control cells without reagent. The next adjacent row of wells contained the lowest concentration of recombinant long acting tissue protective cytokines. Each adjacent row of wells thereafter was filled with sequentially greater concentrations. The cell cultures that were incubated in media with 3% serum and without Epo were plated out at 200,000 cells/ml and 100μl per well in 96-well plates. The contents were mixed briefly and carefully, using the orbital vibrating platform seated on

top of the stir plate. The plate was incubated with different concentrations of the carbamylated ARANESP (from 0.2 pM to 20 nM) for 48 h in RPMI 1640 medium containing 3% serum in a humidified incubator with 5% CO_2 at 37°C. On day four of the assay, the 96-well plate was taken out from the incubator and placed at room temperature in the laminar flow hood. Immediately, the bioactivity is quantified (spectrophotometric absorption at 450 nm, subtracted from background absorption at 620 nm) by measuring the formazan product formed during cellular metabolism of the tetrazolium dye WST1, which correlates with cellular viability/number of cells.

Results

The UT7 cells showed stable and reliable growth in Epo containing media for 3 months.

Neither Carbamylated Aranesp Batch 1 nor Carbamylated Aranesp Batch 2 induced any increased viability of the Epo-dependent UT-7 even at greater than 10,000 pM.

Figure 1 shows concentration-response curves of Epo, Carbamylated ARANESP batch 1 and Carbamylated ARANESP batch 2. Different concentrations of Epo (EPO #5), or two different preparations of carbamylated ARANESP (batch 1 and batch 2) were added to UT-7 cells. Viability was measured 48 h later in the WST-1 assay. Data are mean ± SD of three different experiments each performed in duplicate. The curve is a non-linear regression curve fit. Note that EPO promotes UT-7 proliferation while both batches of carbamylated ARANESP do not.

B. NMDA INDUCED CALL DEATH ASSAY

Excitotoxicity can be defined as the excessive activation of glutamate receptors, such as the N-methyl-D-aspartate (NMDA) receptor. The NMDA receptor exhibits increased activity in response to ischemia and other traumas (Fauci et al., 1998, Harrison's Principles of Internal Medicine), (Nishizawa, 2001, Life Sci. 69, 369-381), (White et al.,

2000, J. Neurol. Sci. 179, 1-33). Thus, the assay serves as a model for assessing a compound's effect on cell injury and death.

Protocol of NMDA excitotoxicity in primary hippocampal neurons

Primary hippocampal neuronal cultures were prepared from new born mice (less than 24 hours old) essentially as previously described by Krohn et al. J. Neurosci. 18:8186-97 (1998). Briefly, the hippocampi were dissected out in DMEM containing 0.02% BSA. The tissue was transferred to DMEM containing 0.1 % papain and incubated for 20 minutes at 37°C. The digestion was stopped by aspiration of the papain containing medium and addition of MEMII and the hippocampal cells were dissociated by trituration with a 1000 µl pipet tip. The tissue pieces were allowed to settle and the supernatant, containing single cells, was transferred into MEMII containing 1% trypsin inhibitor (type II-O) and 1% BSA. The trituration-step was repeated three times before the single cells were centrifuged at 600xg/ minute for 10 minutes and resuspended in growth medium (MEMII, 20mM D-glucose, 100U/ml penicillin, 100 µg streptomycin, 2 mM L-glutamine, 10% Nu-serum (bovine), 2% B27 supplement, 26.2 mM $NaHCO_3$). Cells from 10 hippocampi were used to seed one 24 well plate. One day after seeding, the cells were treated with cytosine-arabino-furanoside (1µM). On day two, the medium was changed and cytosine-arabino-furanoside (1µM) was added.

Excitotoxicity assay

Twelve day old cultures were pre-incubated with test compound (vehicle, carbamylated aranesp) at 0.3 nM for 24 hours. On day 13, the medium was removed from the cells and kept while the cultures were challenged with 300 µM NMDA for 5 minutes at room temperature. After the excitotoxic insult, the pre-conditioned medium was returned to the cultures and the injury was quantified by trypan blue exclusion after another 24 hours of incubation. Approximately 300 neurons were counted per condition in at least four separate wells and the experiments were repeated at least twice (Krohn, A.J., Preis, E. and Prehn, J.H.M. (1998) J. Neurosci. 18(20):8186-8197).

Figure 2 shows that carbamylated ARANESP effectively reduce cell death induced by NMDA when added to the primary hippocampal neuron cell cultures prior to NMDA treatment. Cells treated with vehicle only (control) show little death as assessed

by condensed nuclei. In contrast, cells treated with NMDA (NMDA 300μM) show a three fold increase in cell death. Finally, cells treated with either MK-810 (MK-810 1μM) or carbamylated ARANESP (carb, ARANESP 0.3nM) show significant protection from NMDA-induced cell death.

Example 2

Middle Cerebral Artery Occlusion (MCAO) Studies on Rats.

Male Crl:CD(SD)BR rats weighing 250-280 g were obtained from Charles River, Calco, Italy. Surgery was performed on these rats in accordance with the teachings of Brines, M.L., Ghezzi, P., Keenan, S., Agnello, D., de Lanerolle, N.C., Cerami, C., Itri, L.M., and Cerami, A. 2000 Erythropoietin crosses the blood-brain barrier to protect against experimental brain injury Proc Natl Acad Sci USA 97:10526-10531. Briefly, the rats were anesthetized with chloral hydrate (400 mg/kg-bw, i.p.), the carotid arteries were visualized, and the right carotid was occluded by two sutures and cut. A burr hole adjacent and rostral to the right orbit allowed visualization of the MCA, which was cauterized distal to the rhinal artery. To produce a penumbra (borderzone) surrounding this fixed MCA lesion, the contralateral carotid artery was occluded for 1 hour by using traction provided by a fine forceps and then re-opened. PBS, carbamylated EPO (50 ug/kg-bw, i.v.) and carbamylated ARANESP (50 ug/kg-bw) were administered immediately after the MCAO.

Subsequently, the rats were subjected to behavioral testing. The De Ryck test, set forth in De Ryck et al., Noecortical localization of tactile/proprioceptive limb placing reactions in the rat, *Brain Research*, 573 (1992) 44-60, was performed to assess the limb placement reaction in the treated rats. In accordance with the De Ryck test each of the rats was subjected to six tests. Four tests are measured: how the rat stretches its legs by holding rat 10 cm above a table nose downwards; whether rat keeps in contact with the table by holding rat at the edge of the table and its head at a 45 degree angle; how the rat places the front paws on the table by holding rat at a border of a table and observing whether he places his paws on the table; and measure if the rat "falls" from the table by placing the rat at the edge of a table and seeing whether he lets his front paws fall off the table. The rats were scored as follows 0, no placing; 1, incomplete or delayed placing of

paws, and 2, immediate and complete placing of paws. Two additional tests measured whether the rat goes sidewards on a table by placing the rat parallel to the table and determining whether it attempts to place either front or hind paw on the table and whether the rat falls sidewards from the table by placing rat parallel to an edge of the table and determining if it will let either its front or hind paws fall off table when pushed. The same scoring system was used for these tests but the front and hind paws were graded separately. The maximum score a rat could achieve is 16.

Twenty-four hours after MCAO, the rats were anesthetized as described above and transcardially perfused with 100ml saline followed by 250 ml of sodium phosphate buffered 4% paraformaldehyde solution. Brains were rapidly removed, fixed, and 1-mm brain slices were stained using triphenyl tetrazolium chloride (TTC). M. L. Brines et al., Proc Natl Acad Sci U S A 97, 10526-10531 (2000) hereby incorporated by reference. Infarct volumes were determined by quantitative analysis of the stained brain slices.

Figure 3 shows that carbamylated ARANESP effectively reduce the infarct volume following MCAO. Vehicle-treated (Vehicle) animals had an infarct volume of nearly nearly 250 mm^3. In contrast, a single dose of either carbamylated ARANESP (Caranesp) or carbamylated epo (CEPO) reduced the infarct volume to about 150 mm^3.

Figure 4 shows that carbamylated ARANESP treated rats scored higher on the DeRyck sensor-motor test than the vehicle treated animals. The vehicle treated rats (Vehicle) scored about 3 (out of a maximum of 16) on the De Ryck's test demonstrating an extreme behavioral deficit. In contrast, a single dose of either carbamylated ARANESP (Caranesp) or carbamylated EPO (CEPO) significantly increased the behavioral outcome.

The invention is not to be limited in scope by the specific embodiments described which are intended as single illustrations of individual aspects of the invention, and functionally equivalent methods and components are within the scope of the invention. A skilled artisan would recognize that the results obtained from carbamylated ARANESP would be expected of similar long acting tissue protective cytokines of the present invention. Indeed various modifications of the invention, in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing

description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

All references cited herein are incorporated by reference herein in their entireties for all purposes.

WHAT IS CLAIMED IS:

1. Use of a long acting erythropoietin or a long acting tissue protective cytokine for the preparation of a pharmaceutical composition for protection against an injury or restoration of function following the injury to responsive mammalian cells, tissue or organ.

2. The use of claim 1, wherein the responsive mammalian cells comprise neuronal, brain, spinal cord, retinal, muscle, heart, lung, liver, kidney, small intestine, adrenal cortex, adrenal medulla, capillary, endothelial, testes, ovary, endometrial, or stem cells.

3. The use of claim 2, wherein the cells further comprise photoreceptor, ganglion, bipolar, horizontal, amacrine, Müller, myocardium, pace maker, sinoatrial node, sinus node, atrioventricular node, bundle of His, hepatocyte, stellate, Kupffer, mesangial, goblet, intestinal gland, enteral, endocrine, glomerulosa, fasciculate, reticularis, chromaffin, pericyte, Leydig, Sertoli, sperm, Graafian follicles, primordial follicles, endometrial stroma, and endometrial cells.

4. The use of claim 1, wherein the injury is a seizure disorder, multiple sclerosis, stroke, hypotension, cardiac arrest, ischemia, myocardial infarction, inflammation, age-related loss of cognitive function, radiation damage, cerebral palsy, a neurodegenerative disease, Alzheimer's disease, Parkinson's disease, Leigh disease, AIDS dementia, memory loss, amyotrophic lateral sclerosis, alcoholism, mood disorder, anxiety disorder, attention deficit disorder, schizophrenia, autism, Creutzfeld-Jakob disease, brain or spinal cord trauma or ischemia, heart-lung bypass, chronic heart failure, macular degeneration, toxin induced neuropathy, diabetic neuropathy, diabetic retinopathy, glaucoma, retinal ischemia, or retinal trauma.

5. The use claim 1, wherein the long acting erythropoietin is selected from the group consisting of a chemically modified long acting erythropoietin and a recombinant long acting erythropoietin.

6. The use of claim 1, wherein the long acting tissue protective cytokine lacks at least one activity selected from the group consisting of increasing hematocrit, vasoconstriction, hyperactivating platelets, pro-coagulant activity and increasing production of thrombocytes.

7. The use of claim 6, wherein the long acting tissue protective cytokine is selected from the group consisting of a chemically modified long acting erythropoietin and a recombinant long acting erythropoietin.

8. The use of claims 5 or 7, wherein the chemically modified long acting erythropoietin is selected from the group consisting of

i. A long acting erythropoietin having at least one or more oxidized carbohydrates;

ii. A chemically reduced long acting erythropoietin having at least one or more oxidized carbohydrates;

iii. A long acting erythropoietin having at least one or more modified arginine residues;

iv. A long acting erythropoietin having at least one or more modified lysine residues

v. A long acting erythropoietin having at least one modification of the N-terminal amino group of the erythropoietin molecule;

vi. A long acting erythropoietin having at least a modified tyrosine residue;

vii. A long acting erythropoietin having at least a modified aspartic acid or glutamic acid residue;

viii. A long acting erythropoietin having at least a modified tryptophan residue;

ix. A long acting erythropoietin having at least one amino acid removed;

x. A long acting erythropoietin having at least one opening of at least one of the cystine linkages in the long acting erythropoietin molecule; and

xi. A truncated long acting erythropoietin.

9. The use of claims 5 or 7, wherein the long acting tissue protective cytokine is a long acting recombinant erythropoietin comprising a long acting erythropoietin mutein of one or more altered amino acid residue between position 11 to 15 of SEQ ID NO:5 [SEQ

ID NO:1], position 44 to 51 of SEQ ID NO 5 [SEQ ID NO:2], position 100-108 of SEQ ID NO 5 [SEQ ID NO:3], or position 146-151 of SEQ ID NO 5 [SEQ ID NO:4].

10. The uses of claims 1, 8 or 9, wherein the long acting erythropoietin is a novel erythropoiesis stimulating protein.

11. The use of claim 10, wherein said novel erythropoiesis stimulating proteins has additional n-linked carbohydrate chains at erythropoietin amino acid residues 30 and 38.

12. The use of claim 11, wherein the novel erythropoiesis stimulating protein has at least one carbamylated lysine residue.

13. The uses of claims 1, 8 or 9, wherein the long acting erythropoietin is a diglycosylated and pegylated erythropoietin.

14. A method for protecting or maintaining the viability of a responsive mammalian cell, tissue or organ comprising administering to a mammal a pharmaceutical composition comprising a long acting erythropoietin or a long acting tissue protective cytokine.

15. A method for protecting or maintaining the viability of a responsive *ex vivo* cell, tissue or organ comprising placing said cell, tissue or organ in contact with a pharmaceutical composition comprising a long acting erythropoietin or a long acting tissue protective cytokine.

16. The methods of claims 14 or 15, wherein the cells comprise neuronal, brain, spinal cord, retinal, muscle, heart, lung, liver, kidney, small intestine, adrenal cortex, adrenal medulla, capillary, endothelial, testes, ovary, endometrial, or stem cells.

17. The method of claim 16, wherein the cells further comprise photoreceptor, ganglion, bipolar, horizontal, amacrine, Müller, myocardium, pace maker, sinoatrial node, sinus node, atrioventricular node, bundle of His, hepatocyte, stellate, Kupffer, mesangial, goblet, intestinal gland, enteral endocrine, glomerulosa, fasciculate, reticularis,

chromaffin, pericyte, Leydig, Sertoli, sperm, Graafian follicles, primordial follicles, endometrial stroma, and endometrial cells.

18. The methods of claims 14 or 15, wherein the trauma or injury is caused by a seizure disorder, multiple sclerosis, stroke, hypotension, cardiac arrest, ischemia, myocardial infarction, inflammation, age-related loss of cognitive function, radiation damage, cerebral palsy, neurodegenerative disease, Alzheimer's disease, Parkinson's disease, Leigh disease, AIDS dementia, memory loss, amyotrophic lateral sclerosis, alcoholism, mood disorder, anxiety disorder, attention deficit disorder, schizophrenia, autism, Creutzfeld-Jakob disease, brain or spinal cord trauma or ischemia, heart-lung bypass, chronic heart failure, macular degeneration, toxin induced neuropathy, diabetic neuropathy, diabetic retinopathy, glaucoma, retinal ischemia, or retinal trauma.

19. The methods of claims 14 or 15, wherein the long acting erythropoietin is selected from the group consisting of a chemically modified erythropoietin and a recombinant erythropoietin.

20. The methods of claims 14 or 15, wherein the long acting tissue protective cytokine lacks at least one activity selected from the group consisting of increasing hematocrit, vasoconstriction, hyperactivating platelets, pro-coagulant activity and increasing production of thrombocytes.

21. The method of claim 20, wherein the long acting tissue protective cytokine is selected from the group consisting of a chemically modified long acting erythropoietin and a recombinant long acting erythropoietin.

22. The methods of claims 19 or 21, wherein the chemically modified long acting erythropoietin is selected from the group consisting of

i. A long acting erythropoietin having at least one or more oxidized carbohydrates;

ii. A chemically reduced long acting erythropoietin having at least one or more oxidized carbohydrates;

iii. A long acting erythropoietin having at least one or more modified arginine residues;

iv. A long acting erythropoietin having at least one or more modified lysine residues

v. A long acting erythropoietin having at least one modification of the N-terminal amino group of the erythropoietin molecule;

vi. A long acting erythropoietin having at least a modified tyrosine residue;

vii. A long acting erythropoietin having at least a modified aspartic acid or glutamic acid residue;

viii. A long acting erythropoietin having at least a modified tryptophan residue;

ix. A long acting erythropoietin having at least one amino acid removed;

x. A long acting erythropoietin having at least one opening of at least one of the cystine linkages in the long acting erythropoietin molecule; and

xi. A truncated long acting erythropoietin.

23. The methods of claims 19 or 21, wherein the tissue protective cytokine is a recombinant erythropoietin comprising an erythropoietin mutein having one or more altered amino acid residue between position 11 to 15 of SEQ ID NO:5 [SEQ ID NO:1], position 44 to 51 of SEQ ID NO 5 [SEQ ID NO:2], position 100-108 of SEQ ID NO 5 [SEQ ID NO:3], or position 146-151 of SEQ ID NO 5 [SEQ ID NO:4].

24. The methods of claims 14, 15, 22 or 23, wherein the long acting erythropoietin is a novel erythropoietin stimulating protein.

25. The method of claim 24, wherein said novel erythropoiesis stimulating protein has additional N-linked carbohydrate chains at erythropoietin amino acid residues 30 and 38.

26. The method of claim 25, wherein the novel erythropoiesis stimulating protein has at least one carbamylated lysine residue.

27. The methods of claims 14, 15, 22 or 23, wherein the long acting erythropoietin is a diglycosylated and pegylated erythropoietin.

28. A composition for transporting a molecule via transcytosis across an endothelial cell barrier comprising said molecule in association with a long acting erythropoietin or long acting tissue protective cytokine.

29. The composition of claim 28, wherein the long acting erythropoietin is selected from the group consisting of a chemically modified erythropoietin and a recombinant erythropoietin.

30. The composition of claim 28, wherein the long acting tissue protective cytokine lacks at least one activity selected from the group consisting of increasing hematocrit, vasoconstriction, hyperactivating platelets, pro-coagulant activity and increasing production of thrombocytes.

31. The composition of claim 30, wherein the long acting tissue protective cytokine is selected from the group consisting of a chemically modified long acting erythropoietin and a recombinant long acting erythropoietin.

32. The compositions of claim 28 or 30, wherein the long acting tissue protective cytokine is a chemically modified long acting erythropoietin selected from the group consisting of

i. A long acting erythropoietin having at least one or more oxidized carbohydrates;
ii. A chemically reduced long acting erythropoietin having at least one or more oxidized carbohydrates;
iii. A long acting erythropoietin having at least one or more modified arginine residues;
iv. A long acting erythropoietin having at least one or more modified lysine residues
v. A long acting erythropoietin having at least one modification of the N-terminal amino group of the erythropoietin molecule;
vi. A long acting erythropoietin having at least a modified tyrosine residue;
vii. A long acting erythropoietin having at least a modified aspartic acid or glutamic acid residue;

viii. A long acting erythropoietin having at a modified tryptophan residue;

ix. A long acting erythropoietin having at least one amino acid removed;

x. A long acting erythropoietin having at least one opening of at least one of the cystine linkages in the long acting erythropoietin molecule; and

xi. A truncated long acting erythropoietin.

33. The composition of claim 31, wherein the tissue protective cytokine is a recombinant erythropoietin comprising a long acting erythropoietin mutein having one or more altered amino acid residue between position 11 to 15 of SEQ ID NO:5 [SEQ ID NO:1], position 44 to 51 of SEQ ID NO 5 [SEQ ID NO:2], position 100-108 of SEQ ID NO 5 [SEQ ID NO:3], or position 146-151 of SEQ ID NO 5 [SEQ ID NO:4].

34. The compositions of claims 28, 32 or 33, wherein the long acting erythropoietin is a novel erythropoiesis stimulating protein.

35. The composition of claim 34, wherein said novel erythropoiesis stimulating protein has additional N-linked carbohydrate chains at erythropoietin amino acid residues 30 and 38.

36. The composition of claim 35, wherein the novel erythropoiesis stimulating protein has at least one carbamylated lysine residue.

37. The composition of claim 28, 32 or 33, wherein the long acting erythropoietin is a diglycosylated and pegylated erythropoietin.

38. The composition of claim 28, wherein the molecule is comprised of a hormone, neurotrophic factor, antibiotic, antiviral, antifungal, peptide radiopharmaceutical, antisense drug, antibody, antiviral, pharmaceutical, or anti-cancer agent.

39. A pharmaceutical composition comprising a chemically modified long acting erythropoietin having at least one responsive cellular protective activity selected from the group consisting of protecting, maintaining, enhancing or restoring the function or viability of a responsive mammalian cell, tissue or organ.

40. The pharmaceutical composition of claim 39, wherein the responsive mammalian cells comprise neuronal, brain, spinal cord, retinal, muscle, heart, lung, liver, kidney, small intestine, adrenal cortex, adrenal medulla, capillary, endothelial, testes, ovary, endometrial, or stem cells.

41. The pharmaceutical composition of claim 40, wherein the cells further comprise photoreceptor, ganglion, bipolar, horizontal, amacrine, Müller, myocardium, pace maker, sinoatrial node, sinus node, atrioventricular node, bundle of His, hepatocyte, stellate, Kupffer, mesangial, goblet, intestinal gland, enteral endocrine, glomerulosa, fasciculate, reticularis, chromaffin, pericyte, Leydig, Sertoli, sperm, Graafian follicles, primordial follicles, endometrial stroma, and endometrial cells.

42. The pharmaceutical composition of claim 39, wherein the chemically modified long acting erythropoietin lacks at least one activity selected from the group consisting of increasing hematocrit, vasoconstriction, hyperactivating platelets, pro-coagulant activity and increasing production of thrombocytes.

43. The pharmaceutical composition of claim 39, wherein the chemically modified long acting erythropoietin is selected from the group consisting of

i. A long acting erythropoietin having at least one or more oxidized carbohydrates;

ii. A chemically reduced long acting erythropoietin having at least one or more oxidized carbohydrates;

iii. A long acting erythropoietin having at least one or more modified arginine residues;

iv. A long acting erythropoietin having at least one or more modified lysine residues

v. A long acting erythropoietin having at least one modification of the N-terminal amino group of the erythropoietin molecule;

vi. A long acting erythropoietin having at least a modified tyrosine residue;

vii. A long acting erythropoietin having at least a modified aspartic acid or glutamic acid residue;

viii. A long acting erythropoietin having at least a modified tryptophan residue;

ix. A long acting erythropoietin having at least one amino acid removed;

x. A long acting erythropoietin having at least one opening of at least one of the cystine linkages in the long acting erythropoietin molecule; and

xi. A truncated long acting erythropoietin.

44. The pharmaceutical composition of claims 43, wherein the chemically modified erythropoietin is further modified by altering one or more amino acid residues between position 11 to 15 of SEQ ID NO:5 [SEQ ID NO:1], position 44 to 51 of SEQ ID NO 5 [SEQ ID NO:2], position 100-108 of SEQ ID NO 5 [SEQ ID NO:3], or position 146-151 of SEQ ID NO 5 [SEQ ID NO:4].

45. The pharmaceutical compositions of claims 43 or 44, wherein the chemically modified long acting erythropoietin is a novel erythropoiesis stimulating protein.

46. The pharmaceutical composition of claim 45, wherein said novel erythropoiesis stimulating protein has additional n-linked carbohydrate chains at erythropoietin amino acid residues 30 and 38.

47. The pharmaceutical compositions of claim 46, wherein the novel erythropoiesis stimulating protein has at least one carbamylated lysine residue.

48. The pharmaceutical compositions of claims 43 or 44, wherein the long acting erythropoietin is a diglycosylated and pegylated erythropoietin.

FIG. 1

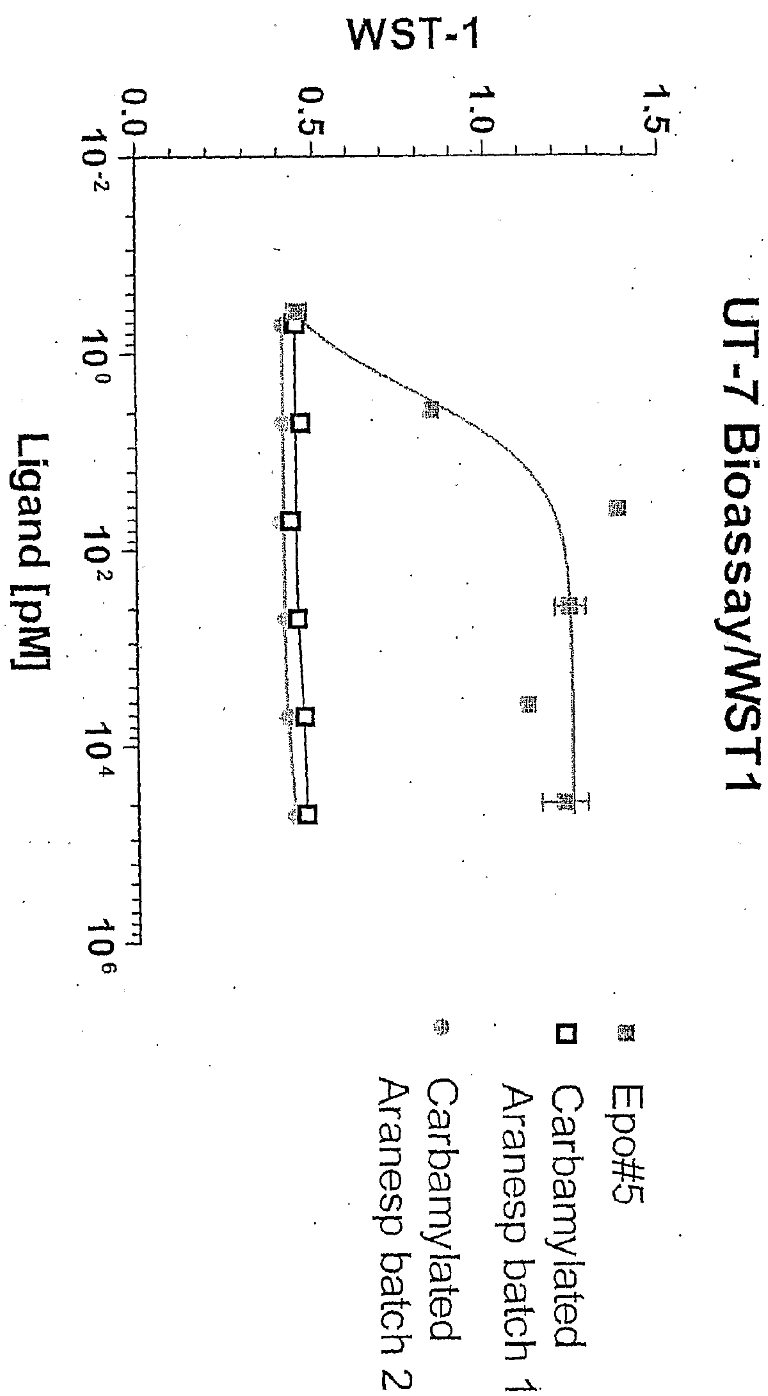

UT-7 Bioassay/WST1
WST-1
0.0
0.5
1.0
1.5
10^{-2}
10^{0}
10^{2}
10^{4}
10^{6}
Ligand [pM]
Epo#5
Carbamylated Aranesp batch 1
Carbamylated Aranesp batch 2

FIG. 2

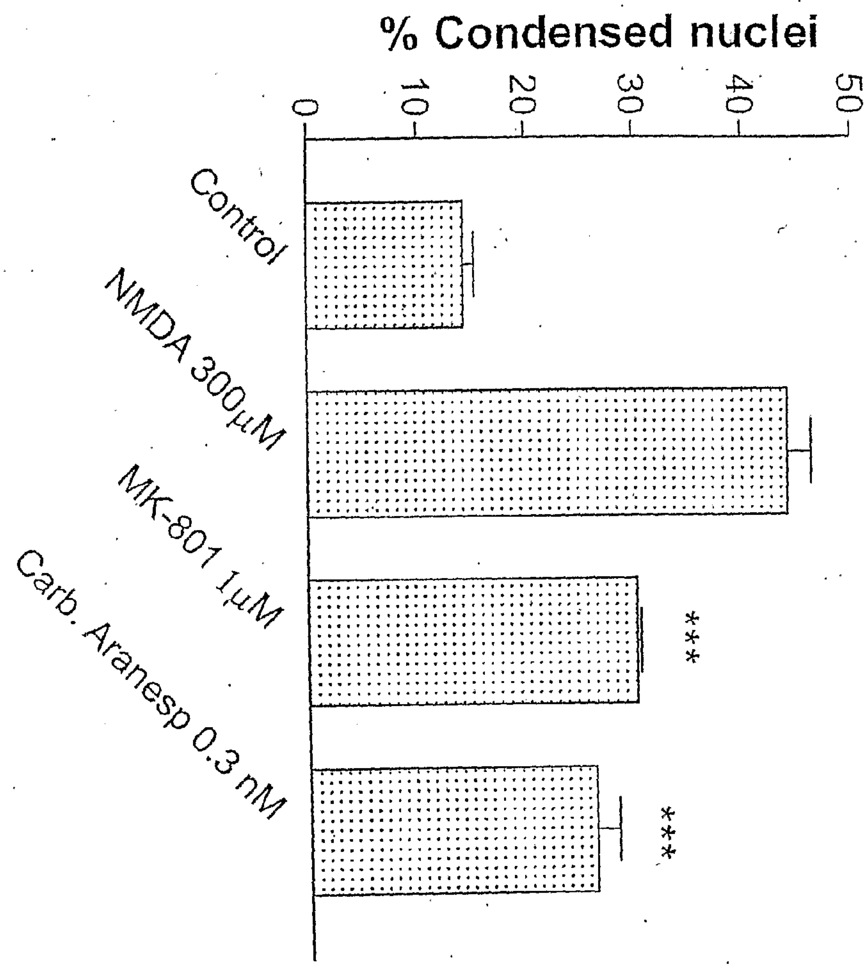

% Condensed nuclei
0
10
20
30
40
50
Control
NMDA 300μM
MK-801 1μM
Carb. Aranesp 0.3 nM
***
***

FIG. 3

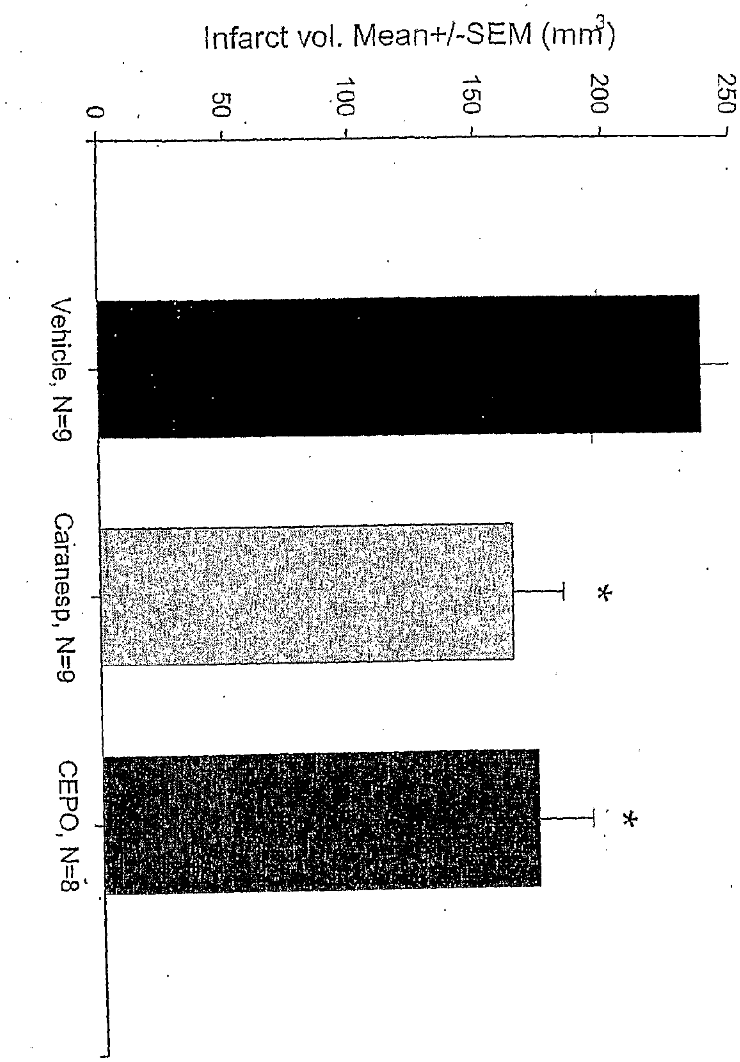
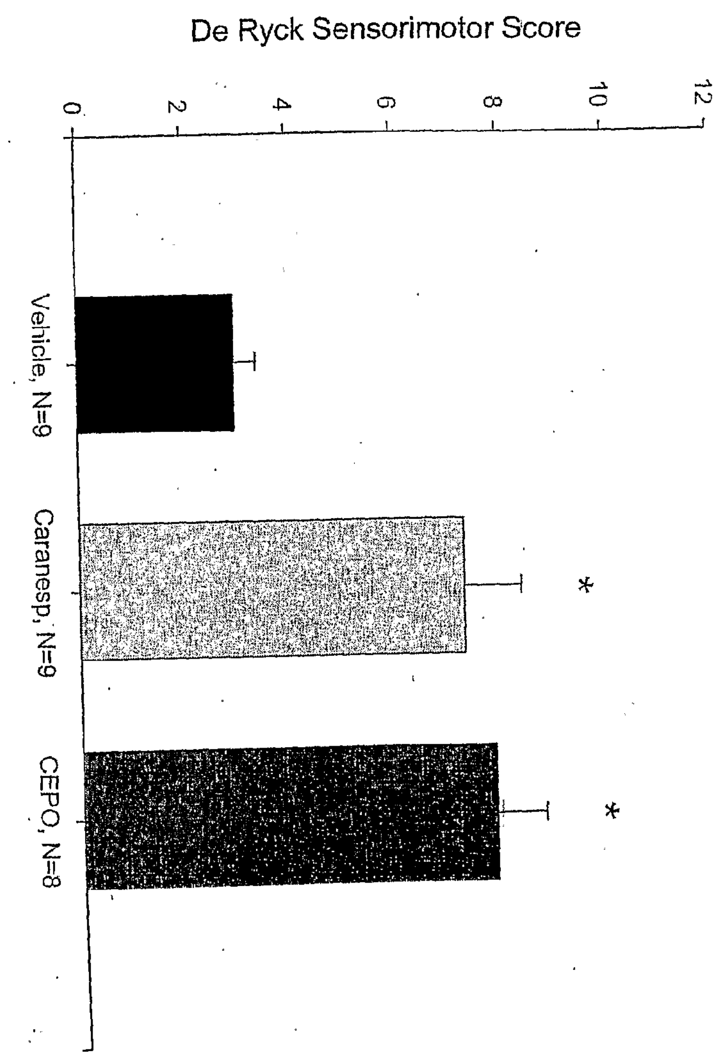

Infarct vol. Mean+/-SEM (mm3)
0
50
100
150
200
250
Vehicle, N=9
Caranesp, N=9
CEPO, N=8
*
*

FIG. 4


De Ryck Sensorimotor Score
0
2
4
6
8
10
12
Vehicle, N=9
Caranesp, N=9
CEPO, N=8
*
*